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CX. A NOTE ON THE PRESENCE OF GLUTATHIONE IN THE CORPUSCLES OF MAMMALIAN BLOOD.

By HENRY FRANCIS HOLDEN (*Beit Memorial Research Fellow*).

From the Biochemical Laboratory, Cambridge.

(*Received July 18th, 1925.*)

THE work to be described was undertaken with the intention of investigating the optically active substances of blood other than glucose.

It was stated by Winter and Smith [1922] that the optical rotation of a concentrated protein-free filtrate of mammalian blood was initially less than that calculated from the amount of glucose estimated to be present. This difference they attributed to the presence of "an unstable modification of glucose."

The writer observed that protein-free filtrates of the corpuscles of sheep and ox blood were free from glucose but contained a laevo-rotatory substance in significant amounts and that its rotation decreased with increase in the hydrogen ion concentration of the solution, thus affording a possible explanation of the phenomena recorded by Winter and Smith.

It was decided to attempt to isolate and identify this substance. The process was necessarily tedious since the optical properties threw little light on its chemical nature.

After numerous attempts with a variety of precipitating agents the following method was employed.

Four litres of sheep's blood were freed from protein with tungstic acid [Folin and Wu, 1919], and the precipitate well washed with cold water. To the united filtrate and washings were added 35 cc. of 10 % mercuric sulphate in 5 % sulphuric acid for each litre of fluid. The precipitate was filtered off and well washed with cold water. It was decomposed with hydrogen sulphide and the sulphuric acid exactly removed with baryta. The filtrate was concentrated under diminished pressure to 50 cc. and enough sulphuric acid added to make the solution semi-normal. Phosphotungstic acid dissolved in semi-normal sulphuric acid was added till the maximum precipitate was obtained. The precipitate was filtered off, well washed, and decomposed with baryta: the barium tungstate was removed by filtration and the excess baryta removed with sulphuric acid. The solution was concentrated to a syrup and on addition of alcohol a white powder was precipitated.

The specimen thus obtained proved to contain both "neutral" sulphur and amino nitrogen. After reduction it yielded the nitroprusside reaction. It had the same melting point as glutathione from other sources and when mixed with a specimen of pure glutathione from yeast the melting point was unchanged. After acid hydrolysis characteristic crystals of glutaminic acid hydrochloride were obtained.

No trace could be found of it in sheep's plasma, which is in accordance with Arnold's [1911] and Hopkins' [1921] earlier experiments using the nitroprusside reaction. Hopkins found that phosphotungstic acid precipitates gluthathione if the latter is in a moderately concentrated solution. He accordingly worked at a low concentration, whilst in the present case the solution was previously concentrated in order to assist the precipitation.

Subsequent experiments indicate that it is present in the corpuscles of sheep, goats, rabbits and rats in the reduced form. Its presence was apparently overlooked by Tunnicliffe [1925].

The yield of 0·05 g. per litre obtained is probably considerably below the actual concentration, since the methods of precipitation involve a considerable loss.

It may be of interest to note that both cysteine and reduced glutathione have the power to convert methaemoglobin into oxyhaemoglobin and to reduce the latter to haemoglobin *in vitro*. This may afford an explanation of the rapid conversion of methaemoglobin into oxyhaemoglobin in the animal body.

Summary.

Glutathione has been identified as a constituent of the red corpuscles of sheep's blood.

It apparently exists in the corpuscles of many animals in the reduced form.

It is apparently the chief optically-active constituent of de-proteinised blood other than glucose and as such is responsible for the effects recorded by Winter and Smith and others and quoted as evidence in favour of the presence of an unstable modification of glucose.

It also probably accounts for much of the "neutral" sulphur of blood.

The writer desires to record his gratitude to Sir F. G. Hopkins for his continual interest and advice.

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CXI. A NOTE ON AN IMPROVED TECHNIQUE FOR USE WITH SYNTHETIC DIETS.

By GLADYS ANNIE HARTWELL.

From the Physiological Laboratory, Household and Social Science Department, King's College for Women, Kensington, London, W. 8.

(*Received July 21st, 1925.*)

The usual method of making synthetic diets is to mix all the dry constituents together and then add sufficient liquid to form a stiff dough. It appears to me that there are many drawbacks to this method of procedure. Primarily, such a mixture bears no resemblance at all to the ordinary articles of food. It is much too concentrated and does not contain anything like the quantity of water usually found in foods (the common foods vary from 40–90 % in water content, e.g. meat 40–70 %, bread 40 %, vegetables 76–96 % [Plimmer, 1921]). In consequence the animals are being fed too small a bulk.

About a year ago, I started using synthetic diets, having previously fed rats on different foods using white bread as a basis. I was immediately struck by the difference in general condition of the animals. When on a synthetic diet the rats were limp to feel, whereas the animals fed on ordinary foods were far fitter and their muscles of better tone. Also the rats fed on the synthetic diet became pot-bellied and had flaccid abdominal walls; some died and *post mortem* examination showed a dilated large intestine, frequently with gaseous contents. I understand from other researchers that this condition is usually present in animals fed on synthetic diets and that nutrition workers have attempted to remedy it by adding some form of "roughage," such as agar, filter paper, charcoal, etc. to the diet. Even with such addition I feel that the bulk taken by the animal is far too small as compared with the normal consumption of food. Therefore, I tried to devise some method by which more water could be incorporated in the synthetic food, so that its composition should approximate more closely to that of an ordinary mixed diet. It was impossible to add more water with the present method because on standing the liquid separated out on top. The next attempt was to add more water to the diet and heat in a saucepan, stirring well until the starch grains burst and so thickened the mixture. By this means no water separated out and a diet prepared in this way gave excellent results when fed to rats. Details of the preparation are given below.

Experimental.

(*a*) *Foods.*

The composition of the diet used in these experiments was as follows:

20 g. caseinogen.
64 g. starch.
4 g. salt mixture [Hartwell, 1922].
12 g. butter.
2 g. yeast extract (marmite).

The caseinogen, starch and salt mixture were ground together in a mortar in bulk. The butter and marmite were added to the ration daily, so that the food was freshly made. The marmite was used as a 5 % solution. This diet may be adversely criticised as being too low in vitamin B, since the majority of nutrition workers in England use 5 g. marmite in 100 g. synthetic diet. The experiments quoted here form part of a series of experiments now in progress to determine the relative amounts of vitamin B required by the lactating and non-lactating rat, and as yet no data have been given for the minimum vitamin B needs of the rat (except by Osborne and Mendel [1922] who find that at least 200 mg. of compressed yeast per day are necessary for it to attain its full weight). In these experiments the average daily intake per animal was 0·2 g. marmite for a rat weighing about 40 g., whilst with older animals the marmite ration was as much as 0·5 g. per day.

Lemon juice was not added to the diet, because it was desirable that the marmite should be the only source of vitamin B. The antiscorbutic vitamin has been omitted in previous experiments of the author's without any detrimental results [Hartwell, 1924], and Simmonds [1924] states that "the rat is apparently immune to scurvy and does not require this vitamin in its diet."

The two diets were prepared as follows:

Method 1. 22 g. of the dry constituents, 10 cc. of 5 % solution of marmite, 6·5 cc. distilled water and 3 g. butter (melted) were beaten together to form a stiff dough.

Thus the total liquid used with 22 g. solid was 16·5 cc.

Method 2. A double saucepan was used. 22 g. of the dry constituents were mixed to a smooth paste with warm distilled water (about 40°), the 10 cc. of marmite solution and the 3 g. butter (melted) were then added and the whole well mixed. Then more distilled water (40°) was added, the total amount used being 65 cc. to the 22 g. of dry solid. This mixture, in the inner saucepan, was then placed in the outer pan containing boiling water and stirred rapidly until it thickened, after which it was left for 1 minute before being removed from the gas. When made in this way, the synthetic diet resembles a rather firm blancmange. The time required for thickening, $2\frac{1}{2}$–$3\frac{1}{2}$ minutes, is too short for any destruction of vitamins to take place. Very hot water cannot be used or the mixture becomes lumpy.

Thus for 22 g. dry food in Method 1, 16·5 cc. liquid were used, whilst in Method 2, 75 cc. were needed. Microscopically this "cooked" food differs from the "raw" mixture in that the starch grains are swollen and the majority are broken.

The animals were not given a definite ration, but were fed so that small quantities were left.

(*b*) *Experiments.*

Exp. 1. *Method of preparing diet changed part way through the experiment.* Twelve rats, 6 ♀ and 6 ♂, weighing about 40 g. each, were given the synthetic diet in the usual form, *i.e.* mixed to a stiff dough. After 19 weeks on this diet the method was changed and the food "cooked" as described above. The experiment was continued for 17 weeks longer.

Exp. 2. *Method of preparing the diet kept constant,* but two groups of animals used, one group being fed on the "raw" and one on the "cooked" food.

A. Rats weighing approximately 40 g. at the beginning of the experiment; 12 animals, 6 ♀ and 6 ♂, fed on each diet.

B. Rats weighing approximately 70 g. at the beginning of the experiment; 8 animals, 4 ♀ and 4 ♂, fed on each diet.

RESULTS.

1. *Growth.* At first the rate of growth of all the rats was equal but after a few weeks the growth curve of those fed on the "cooked" diet was better than that of those fed on the "raw." The average weights of the male rats at the beginning and end of the experiments are given below. (The average weights of the does cannot be given because they became pregnant at different times.)

	Average weight at beginning of experiment	Average weight at end of experiment (10 weeks)	Diet
Exp. 2. A.			
	39 g.	215 g.	"Cooked"
	39	197	"Raw"
Exp. 2. B.			
	69 g.	242 g.	"Cooked"
	68	229	"Raw"

In Exp. 1 the growth curve improved when the "cooked" method was adopted.

2. *General condition of the animals.* The difference in the condition of the two groups of rats was quite obvious. From the first the rats fed on the "cooked" diet were the better animals. They were firm to feel, and equal in all respects (except rate of growth) to the control animals fed on a mixed diet of kitchen scraps, supplemented with bread and milk. The rats fed on the "raw" synthetic diet were limp and pot-bellied. At one period their fur came out, but eventually grew again. This loss of fur (thought to be due to some

dietetic deficiency [Boas, 1924; Hartwell, 1924, 1925]) did not occur at all in any of the rats fed on the diet prepared by Method 2; but every animal on the "raw" diet (three experiments with 32 rats) lost some of its fur and showed bald patches for a week or two. The fact that these differences occurred with diets of the *same* composition, but prepared differently suggests that the "cooked" food can be metabolised more effectively in the animal's body.

3. *Post mortem examinations.* These were made by Miss W. M. Clifford who had no knowledge of the diets used.

The rats fed for the whole experiment on the "cooked" diet were normal when compared with those fed on an ordinary mixed diet, whilst those fed on the "raw" diet had a very enlarged caecum and distended large intestine, with prominent lymphatics.

SUMMARY.

A method is described for the preparation of synthetic diets so that the composition and texture approximate more closely to those of ordinary foods.

Evidence is given that rats fed on a diet prepared by this method grow more quickly and are in better condition than others fed on the same synthetic diet mixed to a stiff paste with water in the usual way.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CXII. NOTE ON THE EFFECT OF HIGH TEMPERATURES ON THE ACCESSORY FOOD FACTOR CONTENT OF COD-LIVER OIL.

By HERBERT WILLIAM SOUTHGATE,

From the Pharmacology Department, The University, Sheffield.

(*Received July 27th, 1925.*)

The experimental work described in this paper was undertaken because of the result obtained on incidentally testing a sample of heated cod-liver oil for its content of vitamin A. This sample of oil had been used to see whether it contained any volatile constituent up to a temperature of 300° at a pressure of 0·2 mm. The pressure above the oil was reduced to this figure before heat was applied to the surrounding oil bath. Then the temperature of the flask contents was raised slowly to 300°, this occupying a period of 6 hours. The flask contents were immediately cooled, air being excluded. Incidentally this oil was tested on rats, and it was found that even 1 g. added daily to the basal diet failed to show any evidence of the presence of the growth factor. Further, X-ray examination of the bones showed that the calcifying principle had also been destroyed during the process. This experiment indicated that heat alone, apart from any external oxidation, could bring about the destruction of these factors—if distinct. A series of experiments, which will be described later, was therefore carried out to determine the effect of heat alone at a lower level on the vitamin content of cod-liver oil.

The stability of vitamin A to heat and oxidation has been the subject of several investigations. The discrepancies between the results obtained by earlier workers in this field were shown by Hopkins [1920] to be due to the fact that the growth-promoting factor is easily destroyed by the oxygen of the air. He showed, on the other hand, that if butter be heated, air being carefully excluded, its vitamin A content is not much reduced by a temperature of 120° maintained for 12 hours.

More recently the view of E. Mellanby [1918] that the anti-rachitic factor is probably identical with vitamin A, has been attacked on the ground that the two principles are not equally stable when exposed to heat and oxidation, the former factor proving more resistant to these processes [McCollum, Simmonds, Becker, and Shipley, 1922; Goldblatt and Zilva, 1923].

In consequence of these results it was decided to determine the effect of heat alone for varying periods on the vitamin content of cod-liver oil. It has

already been stated that the author found that slowly heating cod-liver oil to 300°, air being rigidly excluded, destroyed the growth-promoting and calcifying factors. A lower temperature, 200°, was therefore decided upon. A sample of unrefined cod-liver oil was obtained and 200 g. of this placed in each of three long-neck round-bottomed flasks fitted with a rubber cork having inlet and outlet tubes and a thermometer. The outlet tubes were connected up with tubing, the other end of which dipped under mercury, thus excluding air, but allowing escape through expansion in the flasks. By means of the inlet tubes the flasks were flushed out with dry carbon dioxide before heating was commenced. The flasks were heated in an oil-bath. The temperature of the flask contents was gradually raised to 200°, this process taking about 1 hour. One flask was thereupon disconnected and cooled, air being excluded. This specimen will be referred to as Heated 1 sample. The temperature of the oil in the other two flasks was maintained at 200° for 2 hours, when one of these was disconnected and cooled. The contents of the remaining flask were maintained at 200° for a further 4 hours. The flask was then disconnected and cooled, air being excluded. The last two lots of oil will be referred to as Heated 2 and Heated 3 samples respectively.

Effect on the Growth Curve.

A preliminary testing of these three samples for their content of the growth-promoting factor was then carried out on rats, the basal diet of these animals being: starch 50 %, caseinogen 20 %, oxidised heated olive oil 20 %, marmite 5 %, salt mixture 5 % [McCollum, 1917]. It was found that 5 mg. of the unheated sample restored normal growth when this had ceased on the basal diet. About 40 mg. of the Heated 1 sample, and 180 of the Heated 2 sample were required for this purpose, whilst 0·3 g. of the Heated 3 sample gave a very flat growth curve. It is curious to note that while the rats did not grow much on the last sample, yet in no case did they show xerophthalmia.

In Fig. 1 are illustrated the growth curves of a family of ten rats, pairs of which were being tested preventatively with the same amount (namely, 0·1 g. per rat per day) of the unheated and heated specimens of oil, one pair remaining on the basal diet for control. Similar results were obtained with two other sets of rats.

It will be seen that the growth curve given by 0·1 g. of Heated 1 sample is normal, that the growth produced by the same amount of Heated 2 sample is somewhat below normal, whilst the animals fed with 0·1 g. of Heated 3 specimen put on weight extremely slowly.

Relative calcification effects produced by the samples.

With the diet given, great differences in the calcification of bones were not to be expected. The bones were examined radiographically and their calcium content subsequently estimated. The radiograms of the respective

sets showed that the bones of the rats on the Unheated and Heated 1 specimens were well and equally calcified. Those of the rats on the Heated 2 specimen showed some osteoporosis, which was more marked in the case of those on the Heated 3 specimen. The calcification in this last case showed a slight improvement on that in the bones of rats on the basal diet only.

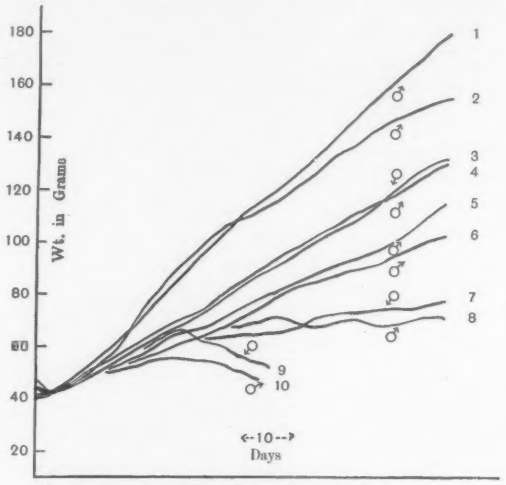

Fig. 1. Rats 1 and 3. 0·1 g. unheated oil
" 2 " 4. 0·1 g. heated 1 sample
" 5 " 6. 0·1 g. " 2 "
" 7 " 8. 0·1 g. " 3 "
" 9 " 10. Basal diet only

The calcium content of the femora of the respective animals worked out as follows:

First set of rats

Specimen of oil	% content of moisture	% content of calcium	
		Wet bone	Dry bone
Unheated			
1st rat	25·7	17·0	22·9
2nd rat	23·1	15·9	20·4
Heated 1			
1st rat	22·0	15·1	19·4
2nd rat	23·5	15·0	21·2
Heated 2			
1st rat	28·3	12·6	17·8
2nd rat	29·7	11·9	17·0
Heated 3			
1st rat	31·6	10·3	15·1
2nd rat	31·8	9·0	14·7
Basal diet only			
1st rat	31·2	10·0	15·5
2nd rat	33·6	8·5	12·6

The bones of the other two sets of rats were not analysed for calcium content.

Thus it will be seen that the calcium estimations bear out on the whole the results found radiographically; that is, there is a gradual disappearance of the anti-rachitic vitamin when the oil is heated to, and maintained at, this high temperature.

Summary of Results.

1. When cod-liver oil is slowly heated out of contact with air to 300° the anti-rachitic and growth-promoting factors contained in it appear to be completely destroyed.

2. If cod-liver oil is slowly heated to 200° and maintained at this temperature for some hours these two factors slowly disappear *pari passu*.

3. Oxidation from external sources is obviously unnecessary for the destruction of these factors at these temperatures.

4. If oxygen be excluded the chemical compound or compounds representing these factors are fairly stable to heat.

The work described above was carried out during the first half of last year, and the main results reported in June, 1924, to the Science Committee of the British Medical Association to whom grateful acknowledgment is made for the grant which defrayed the expenses of the work. Since then Steenbock and his collaborators [1924] have published the results which led them to the conclusion that the presence of the anti-rachitic factor is necessary for the manifestation of growth also.

The possibility of the destruction of the factors in the above experiments being due to the action of the fatty acids liberated by hydrolysis of the glycerides at those high temperatures was one which the author considered and tried to avoid by heating some oil with a weak base out of contact with air. It was found however that as weak a base as magnesium oxide only increased the rate of hydrolysis with much soap formation.

Finally I would express my thanks to Professor Edward Mellanby for his support throughout the work.

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CXIII. THE EFFECT OF ALCOHOL, UNDER VARYING CONDITIONS OF DIET, ON MAN AND ANIMALS, WITH SOME OBSERVATIONS ON THE FATE OF ALCOHOL IN THE BODY.

By HERBERT WILLIAM SOUTHGATE.

From the Pharmacology Department, University of Sheffield.

(*Received July 27th, 1925.*)

The relationship of the alcohol concentration in the blood of animals to varying conditions of the food content of the alimentary canal was first put on a broad experimental basis by the work of E. Mellanby [1919]. This worker used mainly dogs for his investigations in which the alcohol blood concentration curve was determined for varying conditions of the food and drink of these animals. A year later [1920] he described further results obtained with a human subject. Previous to this Weissenfeld [1898] noted that the changes produced by the ingestion of alcohol in his respiratory and circulatory system were greatest when the beverage was taken on an empty stomach. Voltz, Baudrexel and Dietrich [1912] gave moderate doses of alcohol to men and found that the content of alcohol in the urine was six times greater when the alcohol was taken on an empty stomach than when its ingestion had followed the taking of food. Hanzlik and Collins [1913] studied the absorption of alcohol from the alimentary tract of cats and dogs. Though these investigators were not primarily concerned with the influence of foodstuffs on alcohol absorption, they showed that substances such as olive oil, cholesterol and bile salts exerted a retarding effect on alcohol absorption. Vernon [1919] carried out experiments on the influence of alcohol on manual work and neuro-muscular coordination and concluded that at the height of its influence alcohol is about twice as active in upsetting neuro-muscular coordination when taken on an empty stomach as when taken with food. McDougall and Smith [1920], working independently, corroborated Vernon's results. The extensive results published from the Nutrition Laboratory of the Carnegie Institution of Washington have dealt in the main with the psychological effects of alcohol, but among these records are some which illustrate the effect of food on alcohol ingestion. Thus Miles [1924] showed that when a dose of alcohol was taken shortly after the ingestion of a full meal, the effect, as exhibited in the accuracy and speed of neuro-muscular processes involved in typewriting during a 4-hour interval following the ingestion, was very much reduced compared with the effect when no food had been taken. With the exception of Mellanby the above-mentioned authors

have not dealt mainly with the effect of foodstuffs *qua* foodstuffs on the alcohol blood concentration. The work to be described is a more detailed study of the relation of alcohol to diet in the human subject. Three men, who were moderate drinkers of alcoholic beverages, were chosen. They were J. H. B. age 53 years, weight 11st. 4 lb., an army pensioner after 23 years in the R.F.A. who continues at his trade as saddler; G. H. N. age 31 years, weight 11 st. 8 lb. and his brother W. N. age 34 years, weight 9 st. 8 lb. were foundrymen, but for the past 18 months have been unemployed. The investigations covered the period July, 1923–December, 1924. During this interval the weight of the three men remained sufficiently constant to need no change in the amount of alcohol given. Not more than one experiment was carried out per week and the routine was always the same. Their last meal on the day preceding that of the experiment was consumed between 8 and 9 p.m. Alcoholic drinks were, of course, not allowed on this day and no liquid of any kind after 9 p.m. No food or drink was taken before arrival at the laboratory on the morning of the experiment. The dose of alcohol was the same for each man and for each experiment, namely, 1200 cc. of (practically) 8 % alcohol made by adding 300 cc. of absolute alcohol to 3450 cc. of tap water, *i.e.* 96 cc. of absolute alcohol per man. This dose produced little or no evidence of intoxication. There was no difference in their ability to draw a simple diagram, to walk or run, an hour after taking the alcohol from that before the beverage was taken. No flavouring was added to the alcohol, the drinking of which was always spread over 10 minutes. The men rested in the laboratory, the temperature of which was maintained as nearly as possible at 17–18°. In those experiments in which food was taken before the alcohol, the interval between the consumption of the food and taking alcohol was always $1\frac{1}{2}$ hours with the single exception of the period (2 hours) after olive oil. The alcohol content of the blood, urine, etc., was recovered by distillation in the apparatus previously described by the author [1924] and estimated by the slightly modified Pringsheim's method described therein. Blood samples (about 10 g.) were taken at the following intervals after the completion of drinking the alcohol, 1, $2\frac{1}{2}$ and $6\frac{1}{2}$ hours. In several of the experiments the concentration of alcohol in the urine was also determined at intervals. Whether the urine was kept for estimation or not, the times for evacuating the bladder were the same in all the experiments, namely, just before the dose of alcohol, then every 20 minutes during the next $2\frac{1}{2}$ hours, then every $\frac{1}{2}$ hour.

In the accompanying table the concentration of alcohol in the blood is given in cmm. of alcohol per 100 g. blood; the concentration in the urine in cmm. of alcohol per 100 cc. urine. In some of the experiments the whole of the urine for the $6\frac{1}{2}$ hours was pooled and the mean figure for the alcohol content also determined.

The experimental results are set out in the following table:

Table I. *The variation of the alcohol concentration in the blood and urine of the resting subject with varying dietetic conditions.*

		Alcohol concentration in the blood after			Alcohol in urine		Alcohol in urine / Alcohol in blood	
Food taken	Subject	1 hr.	2½ hrs.	6½ hrs.	2½ hrs.	6½ hrs.	2½ hrs.	6½ hrs.
(*a*) No food	J. H. B	146	148	87	193	114	1·37	1·37
	G. H. N.	151	148	78	199	103	1·39	1·34
	W. N.	160	154	85	212	126	1·38	1·40
(*b*) 530 cc. water	J. H. B.	142	145	81	205	127	1·41	1·56
	G. H. N.	130	141	74	199	104	1·41	1·40
	W. N.	170	154	92	197	125	1·28	1·36
(*c*) 530 cc. whole milk previously boiled	J. H. B.	114	130	58	186	87	1·43	1·50
	G. H. N.	123	134	62	184	100	1·37	1·40
	W. N.	Lost	141	71	196	101	1·39	1·42
(*d*) 530 cc. whole milk previously boiled and 183 g. bread	J. H. B.	106	116	48	161	70	1·39	1·46
	G. H. N.	125	116	45	162	58	1·40	1·26
	W. N.	128	123	49	180	82	1·46	1·67
(*e*) 530 cc. water and 183 g. bread	J. H. B.	Lost	125	58	163	83	1·30	1·43
	G. H. N.	137	126	62	164	92	1·30	1·48
	W. N.	159	143	70	191	114	1·34	1·63
(*f*) 50 g. separated milk powder, 183 g. bread, 500 cc. water	J. H. B.	101	117	44	146	67	1·60	1·50
	G. H. N.	140	123	52	154	76	1·25	1·46
	W. N.	157	130	52	182	78	1·40	1·50
(*g*) 50 cc. olive oil	J. H. B.	143	142	79	203	114	1·43	1·44
	G. H. N.	126	146	79	217	125	1·49	1·58
	W. N.	126	155	88	226	128	1·46	1·45

The effect of diet on the concentration of alcohol in the blood (Table I).

(*b*) In these experiments there is no increased rate of absorption when water is previously taken. Mellanby found that a previous drink of water increased the absorption rate of alcohol in the case of dogs.

(*c*) The marked effect of a previous draught of milk in lowering the alcohol blood concentration curve, which Mellanby noted in his experiments is well corroborated.

(*d*) Taking a meal of bread and boiled whole milk previously has an even greater effect than milk alone in lowering the concentration curve which is given by alcohol on an empty stomach.

(*e*) Exps. (*c*) and (*e*) show that the meal of bread and water has about an equal effect to that of whole milk on the subsequent blood concentration curve under the above experimental conditions.

(*f*) This experiment was designed to simulate Exp. (*d*), the milk-fat being absent. The figures in these two experiments agree on the whole quite well. Exps. (*e*) and (*f*) suggest that fats are not the only factor tending to prevent alcohol from circulating in the blood stream.

(*g*) This experiment was carried out to see whether fats as such have any specific effect on subsequent alcohol absorption. In the case of J. H. B. it will be seen that there is little difference between the concentration curves of alcohol in the blood, on an empty stomach, after a drink of water, or after taking olive oil. In the case of the other two subjects there is evidence of delayed absorption at first, but subsequently the concentration curves after

olive oil practically coincide with the respective curves obtained when the alcohol is taken on an empty stomach. On the whole, therefore, it would seem improbable that it is the fat of milk or of bread and milk, as Mellanby [1920] suggested, which is responsible for the alcohol blood concentration curve being so depressed after these foodstuffs.

It is noteworthy that after the maximum concentration of alcohol in the blood has been reached the rate of fall in the concentration of alcohol in the blood is the same for the same individual in all these cases, provided he be kept at rest, and external conditions, especially temperature, be kept constant. This fact was noted by Mellanby in dogs. The results here suggest that within the limit of experimental error this rate of disappearance is the same for all three men, but further experiments will have to be carried out before this can be regarded as established.

This fact of the rate of disappearance of alcohol being independent of the concentration when once the maximum has been reached raises an important question, namely, the fate of a large fraction of the alcohol taken after a meal of bread and milk. For it is obvious from the table that a large fraction of the ingested alcohol after a previous meal of bread and milk never reaches the blood stream, or, if it does, disappears from this at once. The following attempts, unfortunately unsuccessful, were therefore made to trace the fate of this alcohol:

(1) The first solution that suggested itself was that a considerable fraction of the alcohol had never been absorbed, being perhaps adsorbed or retained in some way by the food. To test this, two dogs were given alcohol, one on an empty stomach, the other when a meal of bread and milk had been given $1\frac{1}{2}$ hours previously. Blood samples were taken at intervals; after $2\frac{1}{2}$ hours the animals were instantaneously killed, and the alcohol in the contents of the alimentary canal estimated. The gut wall was not included in this estimation. The results were as follows:

Dog A

Weight	Food taken	Alcohol given	Alcohol in 100 g. blood after		Total alcohol in
15 lb.	No food after 6 p.m. previous evening or water after 8 p.m.	9·75 cc. diluted to 122 cc.	14 mins.	152 cmm.	Stomach 20 cmm.
			44 ,,	184 ,,	Small intestine 0
			85 ,,	175 ,,	Large intestine 20 cmm.
			134 ,,	161 ,,	
			140 ,,	dog killed	
Dog B					
13 lb.	33 g. bread, 250 cc. milk	8·5 cc. diluted to 106 cc.	18 mins.	90 cmm.	Stomach 280 cmm.
			50 ,,	134 ,,	Small intestine 40 cmm.
			116 ,,	131 ,,	Large intestine 110 cmm.
			137 ,,	116 ,,	
			144 ,,	dog killed	

An examination of these figures shows that the amount of alcohol in the lumen of the gut of dog A at the time of killing is practically negligible. From dog B 0·43 cc. of alcohol was recovered. A set of experiments was also carried out to see whether known amounts of alcohol added to bread and milk under

conditions simulating those existing in the stomach and small intestine, could be recovered. It was found that when 10 cmm. alcohol in dilute aqueous solution were added, on the one hand, to 10 g. bread and milk with pepsin and hydrochloric acid, and on the other to 10 g. bread and milk with pancreatic extract and sodium carbonate, and each sample incubated for 2 hours at 37°, the alcohol could be recovered practically completely from both mixtures. It would appear therefore that the alcohol recovered from the contents of the alimentary canal represents the whole of that compound present. If this can be assumed then it is obvious that the presence of 0·43 cc. of alcohol in the alimentary tract of the second dog will not explain fully its low alcohol blood concentration figures compared with those of the first dog.

(2) The low figures for the alcohol content of the alimentary canal of dog A rather rule out the idea that there is a back secretion of the alcohol into the lumen of the gut. Gréhant [1903] found that when he gave large concentrated doses of alcohol intravenously to dogs there was a secretion of this into the stomach. Hanzlik and Collins [1913] found that when alcohol was placed in the stomach or in isolated loops of intestine there was a re-secretion of this compound into the intestine, though the amount was small. Previous to the experiment with the two dogs the present writer had carried out a series of experiments on rabbits to test the degree of back secretion into the alimentary tract. The animals were given no food for 2 days previous to the experiment and their drinking water was withdrawn on the evening preceding the experiment. The alcohol was given intravenously. The following results were obtained:

Rabbit	Weight	Alcohol given	Interval between the alcohol and killing	Concentration in the blood at death	Alcohol found in the alimentary canal
A	5¼ lb.	5 cc. diluted to 50 cc.	25 mins.	208 cmm.	Stomach 75 cmm. Small intestine 35 cmm. Large intestine 46 cmm.
B	5¼	5 cc. diluted to 50 cc.	4¼ hrs.	72 „	Stomach 91 cmm. Small intestine 19 cmm. Large intestine 34·3 cmm.
C	6	—	—	—	Stomach equal to 1·4 cmm. Small intestine 0 Large intestine 1·5 cmm.
D	4¾	4·8 cc. diluted to 48 cc.	8 mins.	258 „	Stomach 39 cmm. Small intestine 45 cmm. Large intestine 56 cmm.
E	5¾	5·8 cc. diluted to 58 cc.	11½ hrs.	3 „	Stomach equal to 2·8 cmm. Small intestine 0 Large intestine 0
F	4¾	4·8 cc. diluted to 48 cc.	5 hrs.	67 „	Stomach 70 cmm. Small intestine 11·7 cmm. Large intestine 44 cmm.
G	5¼	—	—	—	Stomach equal to 2·1 cmm. Small intestine 0 Large intestine 1 cmm.

It is evident that a secretion into the lumen of the gut takes place along its whole length, though not large in amount. It is curious to note here, as in

the case of the two dogs, that the small intestine contains least alcohol. This seems to point to one of two possibilities; either the absorption of alcohol is most rapid from the small intestine [cp. Hanzlik and Collins, 1913] or alcohol is being used up in this part of the tract. It will also be noted that in the case of two control rabbits C and G which were not given alcohol, the amount of volatile reducing substance obtained from the contents of their alimentary tract was negligible in spite of fermentation taking place among the contents of the never empty stomach of the fasting rabbit.

(3) The possibility that the disappearance of the alcohol might be due to some interaction or oxidation taking place during the processes of digestion or bacterial action had to be considered and tested. The following experiments were made.

(*a*) To test the effect of enzymes, well-stoppered bottles containing respectively the quantities given below were incubated for 2 hours at 37°, then the volatile contents were distilled, and the reducing substances in the distillates estimated:

Bottle 1. 10 g. bread and milk, pepsin and hydrochloric acid to make 0·2 %.
„ 2. „ „ ground up stomach mucosa and HCl.
„ 3. „ „ liquor-pancreaticus and dilute sodium carbonate.
„ 4. „ „ liquor-pancreaticus and dilute sodium carbonate and ground up small intestine mucosa.

To each of the above bottles 10 cmm. alcohol were added before incubation.

A corresponding set of bottles containing no alcohol was also put up and similarly incubated. The estimations showed that the alcohol had been recovered practically quantitatively from the contents of bottles 1–4 and that the controls contained none.

(*b*) No organism has been obtained from human faeces which could be classed with the acetic acid producing group. On the other hand the production of alcohol from carbohydrates by members of the *B. coli* group, and by *B. welchii* is a well-known fact. The following experimental evidence shows that when dilute alcohol is added to a suspension of faeces and the mixture maintained at body temperature there is a slow disappearance of the alcohol. 100 g. of freshly passed faeces were emulsified with 150 cc. water and then used in quantities of 10 cc. as follows:

Bottle 1. 10 cc. faeces emulsion + 10 cc. water.
„ 2. 10 cc. faeces emulsion + 5 cc. water + 5 cc. 0·4 % aqueous alcohol.
„ 3. Repetition of 2.
„ 4. Repetition of 2.
„ 5. 10 cc. autoclaved emulsion + 10 cc. sterile water.
„ 6. 10 cc. autoclaved emulsion + 5 cc. sterile water + 5 cc. 0·4 % aqueous alcohol.
„ 7. 10 cc. filtered faeces emulsion and 10 cc. sterile water.
„ 8. 10 cc. filtered faeces emulsion and 5 cc. sterile water + 5 cc. 0·4 % aqueous alcohol.
„ 9. 10 cc. autoclaved filtered faeces emulsion + 5 cc. sterile water + 5 cc. 0·4 % alcohol.

The emulsion of faeces used in bottles 5 and 6 was 0·4 % autoclaved for 20 minutes at 125°. The emulsion of faeces used in bottles 7, 8 and 9 was filtered through an "Imperator" candle. This filtrate was found to be sterile as regards ordinary bacteria. The well-stoppered bottles were then incubated at 37° for 4 hours with the exception of bottles 2 and 3 which were withdrawn

after 1 and 2 hours respectively. After incubation a little milk of lime was added to each bottle and the contents distilled. The alcohol discovered or produced was then estimated with the following results:

Bottle 1. Contained no volatile reducing substances.
" 2. 1·4 cmm. (about $\frac{1}{14}$th) of the alcohol added could not be recovered.
" 3. 2·7 cmm. (about $\frac{1}{7}$th) of the alcohol added could not be recovered.
" 4. 5·0 cmm. (about $\frac{1}{4}$th) of the alcohol added could not be recovered.
" 5. Contained no volatile reducing substances.
" 6. Practically the whole of the added alcohol was recovered.
" 7. Contained no volatile reducing substances.
" 8. 2·6 cmm. (about $\frac{1}{8}$th) of the added alcohol could not be recovered.
" 9. Practically the whole of the added alcohol was recovered.

There is a definite though small disappearance of the alcohol added to faeces *in vitro*—whether this process is increased or not in the colon cannot be determined. The result obtained with the contents of bottle 8 suggests that the active agent is either a filter passer or else a ferment. At the same time the possibility of the effect being due to adsorption cannot be excluded by the results obtained above, for the process of autoclaving markedly alters the physical properties of the emulsion. Thus while this evidence of the using up of alcohol by the faeces could only account for the fate of a small fraction of the alcohol ingested, it gives some explanation of the fact that alcohol cannot be found in faeces when a dose has previously been taken [Atwater and Benedict, 1902; Pringsheim, 1908].

The effect of diet on the concentration of alcohol in the urine (Table I).

It will be seen that these results for the urine corroborate and extend the work of Miles [1922], who showed that when 27·5 g. of alcohol, diluted to one litre, were given to human subjects it produced an alcohol concentration in the urine 40–50 % higher than that in the blood during the period 40 minutes to 2 hours after the ingestion of the alcohol. In the present experiments in which 96 cc. alcohol diluted to 1200 cc. were given, a similar increase in the concentration of the urine-alcohol above that of the blood was found at the $2\frac{1}{2}$ and $6\frac{1}{2}$ hour intervals after the ingestion of the alcohol. Some further determinations, not given here, gave a similar figure at the 1 hour interval. It will be seen from the table that the ratios for the $6\frac{1}{2}$ hour intervals are on the whole slightly higher than those for the $2\frac{1}{2}$ hour intervals, though in some of the individual experiments the ratios at these two intervals agree quite well. In the case where bread and water were taken previously to the alcohol (Exp. *e*) the ratios at the longer interval are distinctly higher. It is difficult to suggest a cause for this last result apart from some specific dietary effect.

The main fact which emerges from these results is that the ratio of the alcohol concentration in the urine to that in the blood keeps fairly constant over a considerable period of time after taking the alcohol and represents a value for the urine 40–50 % above that in the blood. The work of Miles showed that the contention of Widmark [1916] and Ambard [1920], that the concentration of alcohol in the urine was identical with that in the blood, only held

for the first half hour after ingestion, but that afterwards, from 40 minutes to 2 hours, the urine concentration was 30–50 % above that in the blood. The present work shows that this figure practically holds for the subsequent 4 hours as well, and with a much larger dose of alcohol and in greater concentration.

The importance of this fact is urged in view of the present need for a more reliable test for drunkenness. From the above results, as well as those of Miles, it would appear that a close approximation to the alcohol concentration in the blood can be obtained from a knowledge of the actual figure for the concentration in the urine. The alcohol concentration in the blood probably gives us the best means of gauging the intoxication of the subject. Widmark [1915] reported the results of examining the urine of 27 individuals arrested for alcoholic intoxication. The results varied from 450 to below 200 mg. of alcohol per 100 cc. of urine. Not all those arrested were suffering from gross intoxication. In the above experiments the figures for the urine concentration for the 2½-hour period varied from 150–225 cmm. per 100 cc. of urine, and there were no signs of intoxication. Some specimens of urine from persons arrested and subsequently convicted for drunkenness in this city gave figures 360–366 cmm. of alcohol per 100 cc. of urine. Further specimens are being examined in the hope of establishing a figure for the concentration which may be of service in estimating intoxication.

Summary.

1. The effect of certain foodstuffs taken previously to alcohol on the subsequent alcohol blood concentration curve in man is shown.

2. Once the maximum concentration of alcohol in the blood has been reached, provided the subject be at rest and external conditions are maintained constant, then the subsequent rate of disappearance of alcohol from the blood is independent of the concentration.

3. The effect of a previous meal of a foodstuff such as bread and milk in depressing the concentration curve is demonstrated. This is shown not to be due to delayed absorption, but to the fact that a considerable fraction of the alcohol ingested is never manifested in the blood stream.

4. Experiments are recorded of the attempts made to trace the fate of this fraction of the alcohol which "disappears."

5. The concentration of alcohol in the urine exceeds that of the blood over a period of 1–6 hours after ingestion by about 40–50 %; the passage of alcohol through the kidney into the urine cannot, therefore, be merely a diffusion process.

6. Under the experimental conditions employed the amount of alcohol excreted in the urine varies from 3 to 5 % of that ingested.

In conclusion I would express my thanks to Professor Edward Mellanby for his support and criticisms throughout the work.

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CXIV. A METHOD FOR THE ESTIMATION OF GLUCOSE IN BLOOD.

By JOHN ALEXANDER MILROY.

From the J. C. White Biochemical Department, The Queen's University, Belfast.

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So many excellent methods are available for the estimation of glucose in blood, that the introduction of another one requires some justification. The following method probably yields less precise results than several others; but has the advantage of simplicity, and also enables one to determine the approximate amount of sugar in blood without the aid of a colorimeter.

The method is based on the fact that nitro-anthraquinonesulphonates are reduced, when heated with glucose in alkaline solution, first to the corresponding hydroxylamine derivatives having an intense green colour [Wacker, 1902] and later to a deep red substance, which is probably an amine derivative.

The red-coloured final product of reduction is only sparingly soluble in water, and consequently the method is only applicable to dilute solutions of glucose.

A number of nitro-anthraquinonesulphonic acids are known, the chief of these being the 1·6-, 1·7-, α- and β-acids of Claus [1882] and the 1·5- and 1·8-acids [Schmidt, 1904]. These acids, especially the 1·6-acid, are decomposed more or less readily when heated with caustic alkalies, and more slowly by alkaline carbonates, the NO_2 radical being replaced by OH [Frobenius and Hepp, 1907]. The 1·5-acid was found to be the most resistant to the action of alkali, and was therefore selected for the following method. It can be easily prepared in the way described by Schmidt. The isolation of the compound is greatly facilitated by filtering it off through the fritted glass filter discs devised by Prausnitz. Filtration through asbestos is a much slower process. The crystalline precipitate thus separated was purified by recrystallisation from water, and dried *in vacuo* at room temperature.

All the nitro-anthraquinonesulphonic acids have the power of precipitating proteins, the most effective in this respect being the 1·6-acid, but the nitro-anthraquinonesulphonic acid cannot be used both as a protein precipitant and as a reagent for the reduction colour test, since its concentration must be the same in the standard glucose solutions and in the blood filtrates.

Of the different nitro-anthraquinonesulphonic acids, the 1·6-acid was found to be the most delicate with respect to the glucose reaction, but the 1·5-acid

is to be preferred for quantitative work for the reason already given. 0·1 cc. of 0·01 % glucose, 0·1 cc. of 0·4 % solution of the 1·5-acid, and 1·0 cc. of 50 % potassium carbonate solution were made up to 5 cc. and heated in the water-bath for 8 minutes, a similar blank experiment with the reagents being carried out concurrently. Under these conditions a faint red tint was just perceptible in the glucose test when compared with the control.

In view of the fact that blood contains organic substances other than glucose, such as uric acid and creatinine, which act as reducing agents in alkaline solution, it was important to ascertain whether these substances reduce nitro-anthraquinonesulphonates under the conditions of the method for the estimation of glucose to be given later. The ratio of the reducing power of glucose to that of uric acid, dissolved as lithium urate, was found to be 2·35 : 1. The reducing power of creatinine was only about one-tenth that of glucose. These statements only hold when alkaline carbonates are used. In the presence of caustic alkalies the reducing powers of creatinine and urate are increased. The amounts of uric acid and creatinine found in blood cannot therefore seriously affect the calculated glucose values. When the percentages of uric acid and creatinine in blood are much higher than normal, the glucose values may be corrected on the basis of the foregoing ratios.

An intense green colour which later passes into a deep red is obtained when normal urine is rendered alkaline with sodium or potassium carbonate and heated with a solution of the 1·5-acid.

Technique of method.

The proteins are precipitated from blood by the tungstic acid method [Folin and Wu, 1918], the blood being therefore diluted 1 in 10. The mixture of diluted blood with sodium tungstate and $2N/3$ H_2SO_4 is allowed to stand for 30 minutes prior to filtration. 5 cc. of the protein-free blood filtrate are measured into a graduated test tube of 15 cc. capacity. 1·5, 1·8, 2·1, 2·5 and 3·0 cc. of 0·02 % glucose are then measured with a normal pipette or micro-burette of 5 cc. capacity, graduated in 0·02 cc., into five numbered graduated test tubes each of 15 cc. capacity and 12 mm. bore. These quantities are in a geometrical series having an approximate common ratio of 1·2 and correspond to 0·06, 0·072, 0·084, 0·1 and 0·12 % glucose in the undiluted blood with the protein-free filtrate of which they may be found to yield a colour match. If necessary the series may be extended in either direction; but, owing to the sparing solubility of the red product of reduction, the method in this form is only applicable to samples of blood containing not more than 0·16 % glucose. In cases of hyperglycaemia it is therefore necessary to use a smaller quantity of blood filtrate, *e.g.* 2·5 cc., and to make allowance for this fact in the calculation.

One cc. of 0·4 % aqueous solution of 1·5-nitro-anthraquinonesulphonic acid, and 2 cc. of a solution of potassium carbonate, containing 50 g. per 100 cc. of solution, are then added to the contents of each test-tube. All the

solutions are then diluted up to 10 cc. with distilled water, and mixed thoroughly. At this stage a small quantity of the sparingly soluble potassium salt of the 1·5-acid may separate as a crystalline precipitate if the temperature of the room be low. The test tubes are immersed in boiling water for 8 minutes, then cooled thoroughly in running water, and the volume of each solution made up to 12·5 cc. In many estimations the volume was simply restored to 10 cc. The red tint of the tube containing blood filtrate is then compared with the colours yielded by the glucose solutions. An approximate estimate of the percentage of blood sugar is thus obtained.

1 cc. of 0·4 % solution of 1·5-nitro-anthraquinonesulphonic acid was found to be sufficient for amounts of glucose up to at least 5 cc. of a 0·02 % solution.

The method can be rendered more accurate by using amounts of standard glucose solution in a geometrical series having a smaller common ratio, *e.g.* 1·1. This procedure was adopted after an approximate result had been obtained by the first method. As a rule only one intermediate glucose standard requires to be prepared.

As regards the degree of accuracy of the method one may infer from the limits of sensitiveness of the colour reaction, on which the method is based, that the minimal error in the estimation of blood sugar in a sample of blood containing 0·1 % glucose is about 4 %. The percentage error would be proportionally greater than this for samples of blood containing less than 0·1 % and less for those containing more than this amount.

If a colorimeter be available, the solution derived from the blood filtrate is finally compared in it with the glucose solution which it most nearly matches. As a rule, the results were found to be most accurate when a slightly higher glucose standard was taken for comparison.

It is important to avoid delay in making the colour comparison, especially when dealing with samples of blood containing as much as 0·16 % glucose, since part of the sparingly soluble red product of reduction gradually separates when the solutions are allowed to stand for several hours.

The sugar was estimated in a large number of samples of blood from normal animals and from animals under the influence of drugs or subjected to operative interference. A few of these results are given in the following table. For purposes of comparison the sugar in each sample of blood was also determined by Folin and Wu's method [1920].

Table I.

Source of blood	% of glucose (new method)	% of glucose (Folin and Wu's method)	Condition of animal
Rabbit	0·178	0·170	Ether anaesthesia
,,	0·334	0·330	Ether and cooling of pancreas
Dog	0·134	0·121	Ether anaesthesia
Cat	0·43	0·40	Ether and adrenaline
Man (normal)	0·084	0·076	After 4 hours' fast
Ox	0·102	0·096	Normal
Cat	0·03	0·026	During convulsions after insulin.

As will be seen from the table the maximal difference between the two methods is about 10 % except in cases of hypoglycaemia, in which the discrepancy is greater.

SUMMARY.

1. A colorimetric method for the estimation of glucose in blood is described.
2. The method gives somewhat higher values for glucose than that of Folin and Wu.
3. At least 1 cc. of blood is required for an estimation.

I wish to express my great indebtedness to Dr A. E. Campbell and Dr J. S. Loughridge for carrying out a large number of analyses.

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CXV. SOME METABOLIC DIFFERENCES, FOLLOWING X-RADIATION, BETWEEN NORMAL RATS AND RATS IMMUNE TO JENSEN'S RAT SARCOMA.

By EDWARD CHARLES DODDS, WILFRID LAWSON and JAMES CECIL MOTTRAM.

From the Biochemical Department, Bland-Sutton Institute of Pathology, Middlesex Hospital, and the Radium Institute, London.

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When rats are inoculated with Jensen's rat sarcoma, it often occurs that some of them fail to grow a tumour. If subsequently tested by re-inoculation from time to time, they are found to be always resistant and are spoken of as "immune" animals. Certain biological differences have been noted between these "immune" animals and normal animals, but up to the present no definite chemical difference has been demonstrated. It is not proposed in this preliminary communication to deal with the literature of the subject.

Since the response to X-radiation of normal animals and animals inoculated with sarcoma had not been worked out, it was thought that an investigation on these lines might yield interesting results.

Numerous analyses on the blood from these two types of animals, X-radiated and not X-radiated, were made for the following constituents: non-protein nitrogen, urea, creatinine, uric acid, amino-acid nitrogen and sugar. All the determinations were made by the colorimetric methods introduced by Folin.

In the first experiments the whole of the animal was exposed to the X-radiation. A galvanometric method of measuring the dose was employed, but it will suffice now to say that about an erythema dose was given in each case, the spark gap being 18 cm. and the screen being $\frac{1}{10}$ mm. aluminium and 3 mm. wood. The animals were killed at intervals of from 1 hour to 9 days after exposure, and the blood collected from the inferior vena cava into pipettes containing weighed quantities of powdered sodium citrate sufficient to produce 3 % citrate in the blood.

Tables I and II give the results obtained using normal animals. All the results are expressed as mg. per 100 cc. of blood.

An examination of these results shows that X-rays produce no effect upon the non-protein nitrogen and urea content of the blood of normal animals.

Table I. *Normal animals not X-rayed.*

	Non-protein N	Urea		Non-protein N	Urea
1.	54·0	—	7.	61·2	41·5
2.	55·9	—	8.	44·4	32·4
3.	57·4	—	9.	31·0	23·0
4.	61·5	—	10.	40·5	30·0
5.	60·0	33·5	11.	40·0	23·1
6.	58·9	34·2	12.	37·5	24·7

Table II. *Normal animals X-rayed.*

	Time X-rayed	Mean galvo. reading in cm.	Killed after exposure	Non-protein N	Urea
1.	40 mins.	8·49	7 hours	57·0	37·5
2.	40 „	8·49	7 „	47·6	35·1
3.	40 „	10·72	31 „	43·8	24·1
4.	40 „	10·72	31 „	49·2	35·5
5.	30 „	5·46	9 days	61·8	31·3

NOTE. With the galvanometer reading 4·2 cm. an exposure of 1·2 hours gives a dose of one rad, which is the exposure required to kill Jensen's rat sarcoma *in vitro*.

Tables III and IV show the effect of radiation on the blood-constituents of the "immune" type of animal, *i.e.* animals previously inoculated with Jensen's rat sarcoma, in which no tumour resulted. The figures for uric acid, creatinine, sugar and amino-acid nitrogen content are not given, since no change was observed on irradiating normal and "immune" rats.

It will be observed that there is a reduction in the blood-urea content of these "immune" animals and that in many cases the non-protein nitrogen content is also slightly reduced.

Table III. *"Immune" animals not X-rayed.*

	Interval between inoculation and killing	Non-protein N	Urea
1.	12 days	60·7	33·8
2.	18 „	63·1	47·4
3.	—	57·2	41·2
4.	—	44·2	36·7
5.	41 days	45·0	—
6.	41 „	60·0	—

Table IV. *"Immune" animals X-rayed.*

	Interval between inoculation and killing	Time X-rayed	Galvo. reading in cm.	Killed after experiment	Non-protein N	Urea
1.	35 days	35 mins.	9·28	1 hour	40·0	14·0
2.	35 „	35 „	9·28	1 „	43·0	12·9
3.	29 „	40 „	10·4	24 hours	48·3	8·5
4.	29 „	40 „	10·4	24 „	45·1	9·0
5.	32 „	30 „	8·87	3 days	57·2	27·7
6.	32 „	30 „	8·87	3 „	52·8	14·4
7.	32 „	30 „	5·1	3 „	44·9	23·6
8.	32 „	30 „	5·1	3 „	—	15·1
9.	44 „	30 „	6·8	3 „	32·2	9·6
10.	44 „	30 „	6·8	3 „	33·7	11·0
11.	44 „	30 „	6·8	3 „	35·7	10·4
12.	44 „	30 „	5·8	3 „	39·7	12·2
13.	44 „	30 „	5·8	3 „	42·8	12·5
14.	44 „	30 „	5·8	3 „	34·1	14·5
15.	57 „	30 „	5·46	9 „	57·2	32·1

On comparing Tables I and III it is seen that "immune" animals do not differ from normal animals in the non-protein nitrogen or urea blood content prior to X-radiation. A difference is, however, to be observed when these two kinds of animals are X-rayed (cp. Tables II and IV). In the "immune" animals there is a marked reduction in the blood-urea content and in many cases a slight reduction in the non-protein nitrogen content, whereas normal animals do not exhibit these changes after similar X-radiation.

These results suggested an inquiry into the behaviour of tumour-bearing animals under similar treatment.

Table V summarises these results.

Table V.

	Urea mg. %
1. Animal with growing tumour not X-rayed	32·7
2. Animal with growing tumour not X-rayed	45·4
3. Animal with growing tumour X-rayed	44·4
4. Animal with growing tumour X-rayed	43·5
5. Animal with disappearing tumour not X-rayed	31·9
6. Animal with disappearing tumour X-rayed	29·1
7. Animal with disappearing tumour X-rayed	64·4

It would appear therefore that tumour-bearing animals behave in the same manner as normal animals.

In all these experiments the whole animal was exposed to the rays. It was thought that the screening of certain organs might lead to information as to the cause of the fall in blood-urea content in "immune" animals, but the experiments showed that there is no particular organ which is especially acted on by the radiation so as to result in a reduced blood-urea content.

Summary.

No difference in metabolism has hitherto been described between normal animals and animals immune to Jensen's rat sarcoma.

The observations described show that there must be some peculiarity in the metabolism of the "immune" animals, since an exposure to X-radiation causes an immediate fall in the blood-urea content, from a normal value of about 35 mg. per 100 cc., to about 14 mg. per 100 cc. This reaction to X-radiation appears to be a permanent property of "immune" animals.

At the present stage, it would be unwise to offer any explanation of these results, but it is hoped to extend the observations to animals rendered immune by artificial means.

CXVI. A DELICATE COLOUR REACTION FOR THE PRESENCE OF VITAMIN A.

By OTTO ROSENHEIM and JACK CECIL DRUMMOND.

From the National Institute for Medical Research, Mount Vernon, Hampstead, and the Department of Physiology and Biochemistry, University College, London.

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In a previous communication [1920] we stated that the well-known colour reaction of cod-liver oil with sulphuric acid is given not only by the liver oil of this fish but by the liver fats of all animals, and suggested a relationship of the chromogenic substance to vitamin A. The close parallel between the growth-promoting factor and the sulphuric acid test was afterwards emphasised by Drummond and Watson [1922] and further experience, extending over three years, has fully convinced us that this association is more than accidental. The transient nature of the reaction unfortunately prevents its use for a quantitative colorimetric comparison of different oils and all attempts to stabilise the reaction were unsuccessful.

In the course of work on the activation of cholesterol by ultra-violet light [Drummond, Rosenheim and Coward, 1925], we found that arsenic chloride (and various other reagents, see below) gives with cod-liver oil a brilliant ultramarine blue colour reaction. Further study of this reaction led us to the conclusion that, like the sulphuric acid test, it is characteristic for vitamin A. It has the advantage that the colour persists sufficiently long to allow a colorimetric comparison with a suitable standard.

The reaction is carried out by adding 1 cc. of pure arsenic chloride to one drop of cod-liver oil, and shaking the test-tube at once. The oil dissolves immediately to form a clear blue solution, which in the course of a few seconds assumes a purple tint and gradually fades. The reaction is characterised by a well-defined absorption band extending from λ 550–590. Under the above conditions the band persists for about 5 minutes.

Sensitiveness. The reaction is extremely sensitive. A 1 % solution in light petroleum of a highly growth-promoting Newfoundland cod-liver oil was still found to react intensely with 1 cc. $AsCl_3$ in amounts of 0·05 cc. = 0·5 mg. of oil. The limit was reached with 5 $mm.^3$ = 0·05 mg., measured with a Wright's capillary pipette. Compared with the ordinary sulphuric acid test in the same solution the sensitiveness was 20 times as great.

The oil was saponified and the unsaponifiable portion freed from cholesterol by means of digitonin. The cholesterol-free fraction now gave the reaction in a dilution of 1 : 2,000,000.

Relation to vitamin A. The fact that the chromogenic substance resists saponification and is concentrated in the cholesterol-free unsaponifiable fraction of cod-liver oil strongly suggests its close relationship to vitamin A, since it has previously been shown by Drummond and Coward [1922] that this fraction contains the fat-soluble vitamin A. This can be distilled with superheated steam in a nitrogen atmosphere, or in a high vacuum, without losing its activity, and still gives, as we found, the colour reaction with $AsCl_3$ in undiminished intensity. On the other hand, the chromogenic substance, and with it the growth-promoting power, is gradually destroyed by oxidation when a current of air is passed through the oil at 100°: after 30 minutes' aeration the reaction is diminished and is no longer given after 60 minutes.

We have further tested a series of over thirty oils and fats and have found complete agreement between the colour intensity and the growth-promoting activity, as tested by animal experiment. Amongst these samples was the butter made from the milk of a cow which had been fed with cod-liver oil. This butter gave the reaction most intensely as compared with ordinary butters, whilst vegetable oils free from vitamin A gave no reaction. Details of this work will be given in a later communication, together with the results of further tests still in progress.

It is interesting to note that this reaction makes it possible to differentiate the growth-promoting vitamin A from the anti-rachitic vitamin D. We have found that cholesterol which had become highly anti-rachitic by irradiation with ultra-violet light [Rosenheim and Webster, 1925] does not react in the way described above with arsenic chloride[1]. We intend to examine in this direction the oils from the marine diatom, Nitzschia, and of plankton, which were found not to respond to the sulphuric acid test by Drummond and Watson [1922], since in the light of more recent experience it is possible that such growth-promoting power as they exhibit may be due to their content of anti-rachitic vitamin and not to the true, growth-promoting, vitamin A.

Experiments which we made on the diffusibility of the fat-soluble vitamins make it highly probable that they both dialyse through a rubber membrane into light petroleum. The oil recovered from the dialysate gives the colour reaction intensely and is also anti-rachitic, as tested by the animal experiment (unpublished experiments by Rosenheim and Webster).

Nature of chromogenic substance. In searching for an explanation of the chemical nature of the reaction, we found that cod-liver oil (and other liver fats) reacted not only with arsenic chloride, but yielded precisely similar blue colour reactions with a number of heterogeneous reagents, which are known to share with arsenic chloride the property of giving a red colour with chole-

[1] The colour reactions of irradiated cholesterol will be described together with its anti-rachitic power in another communication.

sterol. These reagents are dimethyl sulphate, trichloroacetic acid, acetyl chloride and benzoyl chloride (the last two only in the presence of zinc chloride). Whilst these substances react with cholesterol only when heated, and yield a permanent red colour (absorption band in green), they react with cod-liver oil at room temperature, the blue colour produced (absorption band in yellow) fading within 5–10 minutes.

The reaction with trichloroacetic acid is particularly striking and may be carried out either by allowing a drop of cod-liver oil to fall on a few crystals of the acid or by adding 1 cc. of a saturated chloroform solution of trichloroacetic acid to one drop of the oil[1]. The colour produced is slightly more purple than that with $AsCl_3$ and the absorption band is correspondingly shifted to the red. The colour is discharged by alcohol, ether, ethyl acetate, acetic anhydride, glacial acetic acid and 90 % formic acid, but not by benzene, toluene, light petroleum and chloroform. Pure mono- and dichloroacetic acids do not react.

It would be premature to assume from the analogy of these reactions to those of cholesterol that vitamin A is a sterol derivative. The interesting suggestion of Harden and Robison [1923] with regard to the mechanism of the sulphuric acid test and the observations of Whitby [1923] on cholesterol reactions in general, would tempt one to assume as an explanation of the new reactions that they are due to the presence of an aldehydic coupling substance or to a substance allied to cholesterol, possessing an aldehydic group in its molecule. It has indeed been stated by Takahashi [1922] that a fraction of the unsaponifiable matter of cod-liver oil, claimed by him to be vitamin A, possesses aldehydic character, as evidenced by the reduction of Fehling's solution and ammoniacal silver nitrate. We also have found clear evidence for the presence of a reducing substance in cod-liver oil, in so far as it gives Schiff's reaction and reduces phosphomolybdic acid. The reducing substance is destroyed by aeration, but withstands saponification to a considerable extent, and is therefore not likely to be aldehydic. A colorimetric comparison by means of Welmans' [1892] phosphomolybdic acid test moreover convinced us that the amount of the reducing substance in various oils does not run parallel to their vitamin A content, as measured by the animal experiment and by the arsenic chloride reaction. The nature of the reducing substance remains to be investigated.

Another observation we made links the chromogenic substance to the lipochromes, of which carotene especially yields, in light petroleum or chloroform solution, a slate-blue colour with arsenic chloride (and the other reagents). This reaction is distinguished from that of cod-liver oil by being permanent.

In view of the possible relationship between sterols and lipochromes and their general association with vitamin A in plant tissues [Coward, 1923], it is suggested that the arsenic chloride reaction is concerned with a substance

[1] The chloroform solution should be freshly prepared, as it slowly generates phosgene on keeping in light. A light petroleum solution may be used.

derived from these types of synthetic plant products under the influence of sunlight.

Colorimetric estimation of vitamin A. The application of the colour reaction to the evaluation of the vitamin A content of cod-liver oil is still under investigation. Should further work confirm the results, which show, so far in every case examined, that the colour intensity is proportional to the growth-promoting power, it will be feasible to replace the animal experiment to a large extent by a chemical test. Preliminary experiments indicate that even a rough colorimetric comparison with a suitable standard yields results of a higher degree of accuracy than those obtainable by a laborious animal experiment. The presence of large amounts of yellow pigment naturally influences the tint of the reaction, but since modern medicinal oils, the main source of vitamin A, are usually only slightly pigmented there is no difficulty in this respect.

We have so far made use of the arsenic chloride and the trichloroacetic acid reaction, and have taken as our standard of vitamin A a highly active Newfoundland oil. A suitable colour standard for the arsenic chloride reaction is a mixture of 100 cc. crystal violet solution (1 : 10,000) with 50 cc. methylene blue solution of the same strength (both in alcohol). Under the conditions chosen we found that the colour produced by 20 mg. of the oil (= 1 drop from a pointed glass rod of 3 mm. diameter) + 1 cc. $AsCl_3$ matches the standard dye solution diluted in the proportion 3 : 2.

It is advisable to add the reagent by means of a teat-pipette, holding 1 cc., and to make the comparison rapidly within a few seconds after the addition of the reagent. After preparing a set of suitably diluted standard solutions in test-tubes of uniform diameter, we found, after a little experience, no difficulty in ranging all the oils so far examined in a series which agreed with their growth-promoting activity.

One of us (O. R.) is greatly indebted to the Medical Research Council for their permission to work at the National Institute for Medical Research, and to Dr Dale and Dr Dudley for placing the facilities of their department at his disposal.

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CXVII. THE RELATION BETWEEN THE PANCREAS AND THE CARBOHYDRATE METABOLISM OF MUSCLE.

II. ANTIGLYOXALASE AND GLYOXALASE.

By DOROTHY LILIAN FOSTER.

From the Biochemical Laboratory, Cambridge.

(*Received August 3rd, 1925.*)

1. The lactic acid inhibitor and antiglyoxalase.

In a recent paper on the pancreatic control of carbohydrate metabolism in muscle [Foster and Woodrow, 1924] there was described a factor found in the pancreas and capable of inhibiting the production of lactic acid by chopped muscle *in vitro*. It was pointed out that another substance found in the pancreas, namely the antiglyoxalase described by Dakin and Dudley [1913, 4], might have the same effect. According to these authors antiglyoxalase inhibits the action of glyoxalase, thereby preventing or diminishing the production of lactic acid from methylglyoxal and of mandelic acid from phenylglyoxal. If the lactic acid produced by muscle *in vitro* is derived from methylglyoxal by the action of glyoxalase, its formation would similarly be prevented by the addition of antiglyoxalase to the mixture. If, on the other hand, the production of lactic acid from carbohydrate in muscle is not through methylglyoxal, but some other intermediary product, glyoxalase will not form part of the system and therefore antiglyoxalase will not inhibit its production. It is also true that if a certain factor inhibits the production of lactic acid in muscle, but not the production of α-hydroxy-acids from α-ketonic aldehydes, the lactic acid is probably not derived from methylglyoxal but from some other intermediary compound.

The experiments described below were undertaken with the object of deciding whether the factor which inhibits lactic acid formation is identical or not with antiglyoxalase, and hence to get some evidence as to whether or not methylglyoxal is an intermediary product in the carbohydrate metabolism of muscle.

The presence of methylglyoxal has never been demonstrated in the tissues, and its properties render it extremely unlikely that it could persist in sufficient quantities for this to be possible; but there is every reason to believe that, in the liver at any rate, it can form a stage in the breakdown of glucose. This follows from the work of Dakin and Dudley [1913, 2] on the presence of glyoxalase

in the liver and more especially from their perfusion experiments [1914]. There is, however, not the same evidence for its presence in muscle. This will be discussed more in detail in Part III of this paper.

Though all the experimental work to be described has been done on muscle extracts, or chopped muscle, there is every reason to believe that the fundamental chemical reactions are the same as those involved in the production of lactic acid in the living, intact muscle [Meyerhof, 1920, 1921 and Hill, Long and Lupton, 1923].

In the paper referred to above [Foster and Woodrow, 1924] some evidence was brought forward to show that the lactic acid inhibitor and antiglyoxalase are *not* identical and this is confirmed by the work to be described in the present paper.

Experimental details.

The method described by Dakin and Dudley [1913, 3; 1914] for the detection of glyoxalase and antiglyoxalase has been followed exactly, and, unless otherwise stated the procedure has been the same in all experiments. The glyoxalase solution used was obtained by treating minced chicken muscle with five times its weight of water and, after allowing the mixture to stand for an hour, filtering it through muslin. 50 cc. of the glyoxalase solution were allowed to act on 0·2 g. phenylglyoxal dissolved in 10 cc. water. The mixture was incubated over night at 37° with an excess of precipitated chalk and a few drops of toluene in a conical flask. The resulting mandelic acid was extracted with ether and its rotation measured, exactly in the way described by the above authors. When the muscle extract was allowed to act on the phenylglyoxal alone, a relatively large residue of white crystals of mandelic acid was left after the evaporation of the ether. This was dissolved in 15 cc. of water, boiled with a trace of charcoal and filtered. The rotation of the solution was measured in a 2 dm. polarimeter tube and the figures given in the tables are the rotations observed with mercury green light. The solutions examined were always perfectly clear and colourless. When a solution was tested for antiglyoxalase it was added to the glyoxalase some time, usually an hour, before the phenylglyoxal and the mixture kept at 37°. The control mixtures were always similarly incubated with an equal volume of saline.

Foster and Woodrow [1924] showed that a 70 % alcoholic extract of a dried pancreas preparation (the alcohol being subsequently removed by vacuum distillation, etc.) contained the lactic acid inhibitor, and it was an extract prepared in this way which was tested for antiglyoxalase. The same preparation of dried pancreas was used throughout, but the extract was made fresh for each experiment. In some cases (Exps. 2 and 3) it was found that the extract did have some antiglyoxalase action, but by exercising great care in preparing it, in particular by using alcohol accurately diluted to 70 % and centrifuging till the solution was perfectly clear, an extract was obtained which had no inhibiting action on glyoxalase (Exp. 4, Nos. 1 and 4).

Exp. 1. Chicken muscle used as source of glyoxalase. Two pancreatic extracts tested A and B. (B had been prepared some weeks earlier and kept at −7°.)

Table I.

No.	20 % extract of chicken muscle cc.	Calcium carbonate suspension cc.	Inhibitory factor	Phenyl-glyoxal g.	Toluene drops	Rotation	Notes
1	50	10	None	0·2	5	−3·16°	Control
3	50	10	5 cc. A	0·2	5	−3·46°	Duplicate
4	50	10	5 cc. A	0·2	5	−3·16°	No inhibition
5	50	10	5 cc. B	0·2	5	−3·3°	No inhibition
7	50	10	5 cc. A heated to 85° for 10 mins.	0·2	5	−3·01°	No inhibition
9	50	10	5 cc. A boiled	0·2	5	−3·27°	No inhibition
	Incubated 1 hour						
	Incubated 20 hours						

This solution therefore did not contain antiglyoxalase. Some of the same preparation was found to inhibit the formation of lactic acid in pigeon muscle by about 50 %[1].

Exp. 2. All details as in Expt. 1, except that the chicken muscle extract had been stored some time at −7° and had apparently lost some of its glyoxalase as the controls gave low rotations.

Table II.

No.	Inhibitor	Rotation	Notes
1	None	−0·98°	Control
2	None	−1·01°	Control
3	5 cc. 70 % alcoholic extract of pancreas	−0·81°	Slight inhibition
4	„ „	−0·90°	Slight inhibition

This preparation therefore did contain traces of antiglyoxalase.

Exp. 3. A 70 % alcoholic extract of pancreas was tested both for lactic acid inhibitor and for antiglyoxalase and chicken muscle was used in both cases.

A. *Lactic acid estimations.*

In each of four flasks were placed 10 cc. phosphate buffer solution p_H 7·5, 3 cc. saline or pancreatic extract and 1 cc. *N*/10 KCN and 4 g. minced muscle [see Foster and Woodrow, 1924]. Left at room temperature for 24 hours.

Table III.

No.	Inhibitor	% of lactic acid	Notes
1	3 cc. saline	(Lost)	Control
2	3 cc. saline	1·5	Control
3	3 cc. extract	0·911	40 % inhibition
4	3 cc. extract	0·787	47 % inhibition

There is therefore at least a 40 % inhibition of lactic acid production due to the extract.

[1] Unpublished experiment by Mrs D. M. Needham.

B. *Glyoxalase estimations.*

Table IV.

No.	Inhibitory factor	Rotation	Notes
1	6 cc. saline	−2·03°	Control
2	6 cc. saline	−2·12°	Control
3	3 cc. extract + 3 cc. saline each	−1·82°	16 % inhibition
4		−1·64°	16 % inhibition
5	3 cc. insoluble residue + 3 cc. saline each	−1·64°	26 % inhibition
6		−1·42°	26 % inhibition
7	3 cc. extract + 3 cc. insoluble residue	−1·55°	20 % inhibition
8	3 cc. extract + 3 cc. ether-soluble residue	−1·45°	29 % inhibition

Not only was the final purified 70 % alcoholic extract of the pancreas tested for antiglyoxalase, but also the various fractions removed in course of preparation. The figures show that the extract contained some antiglyoxalase, but so did the residues from the ether and centrifuging. The inhibition of the glyoxalase by 70 % alcoholic extract is only 16 % of the total (as against 40 % of the lactic acid), and as the residues gave higher figures it seemed probable that more careful preparation of the final solution would remove all the antiglyoxalase.

A similar but more conclusive result was obtained in Expt. 4 in which a water extract was made of the residue of the gland insoluble in 70 % alcohol. The alcoholic extract was made in the usual way. The residue was then dried in a current of warm air to remove all traces of alcohol, and ground with sand and distilled water and left overnight. Then the mixture was centrifuged till a clear supernatant liquid was obtained. This was made up to 1 % with sodium carbonate and incubated at 37° to remove trypsin, as recommended by Dakin and Dudley [1913, 4]. It was found that there was some trypsin still left, but it has been shown that antiglyoxalase is distinct from trypsin (Dakin and Dudley [1913, 2] and Exp. 7 below). 5 cc. of this extract were equivalent to 1 g. of the dried gland.

Exp. 4.

Table V.

No.	Extract	Treatment	Rotation	Notes
12	5 cc. saline	—	−3·21°	Control
11	5 cc. saline	—	−3·12°	Control
1	5 cc. alcoholic extract	—	−2·92°	Very slight inhibition
2		—	−3·00°	
3	5 cc. alcoholic extract	Heated at 85° for 10 mins.	−3·14°	No inhibition
4			−3·04°	
5	5 cc. water extract	—	−0·52°	Almost complete inhibition
6		—	−0·51°	
7	5 cc. water extract	Heated at 85° for 10 mins.	−2·16°	Some destruction of antiglyoxalase
8			−2·13°	
9	5 cc. water extract	Boiled for 10 mins.	−1·72°	Some destruction of antiglyoxalase
10			−1·64°	

These figures show that the extract made with 70 % alcohol was practically free from antiglyoxalase, whilst the watery extract was rich in this factor and could inhibit the glyoxalase. It would appear therefore that the lactic acid inhibitor is extracted by 70 % alcohol, whilst the antiglyoxalase is not.

Some observations on antiglyoxalase.

In all the experiments with phenylglyoxal it was noticed that whenever there was any antiglyoxalase action, that is to say when no mandelic acid was produced after adding phenylglyoxal to a mixture of muscle extract and an extract of pancreas, there was a development of an orange coloration. The colour produced was always proportional to the degree of inhibition. If this was complete the colour produced during incubation of the mixture was a deep orange, and, with partial inhibition lighter colours were obtained. This phenomenon was noticed whatever pancreatic preparation was used, fresh fowl and ox pancreas, or a watery extract of the dried pancreas; and also when an alcoholic extract showed some inhibitory power, as in Exp. 2. The colour gradually increased during incubation.

The greater part of the colouring matter was precipitated during removal of the protein, by boiling after addition of ammonium sulphate, and the coagulum was always brightly coloured, but some colour persisted in the filtrate. Acidification with phosphoric acid produced no change but the ether solution and residue frequently turned red, and once or twice a blue colour was noticed at this stage. All traces of the colour disappeared on boiling with charcoal and the final solution was as clear and colourless as the controls. The control flasks in which mandelic acid was produced from the phenylglyoxal never showed any trace of this orange colour at any stage. Since this colour was invariably associated with a failure to convert the phenylglyoxal into mandelic acid it seemed worth while to investigate the connection between the two phenomena.

At first sight it seemed probable that the colour might be due to the excess of phenylglyoxal present when the glyoxalase was inhibited; but this can hardly be the case, as in the controls also there must be a large excess of unchanged phenylglyoxal present. A rotation of $-3°$ only means a conversion of 30 % of the 0·2 g. phenylglyoxal added to each flask into mandelic acid. The unchanged excess is precipitated during the boiling with ammonium sulphate [Dakin and Dudley, 1913, 1].

Then it was found that on incubating a pancreatic extract with phenylglyoxal, in the absence of any muscle extract or any other tissue preparation, the mixture turned the same deep orange colour and an orange precipitate was formed. Evidently there was some factor in pancreatic extract which could bring about this change in the phenylglyoxal. Is the apparent "antiglyoxalase" action of pancreatic preparations due, not to their inhibitory action on the glyoxalase, but to the fact that they contain something capable of changing phenylglyoxal into a highly coloured body which cannot give rise to

mandelic acid? In other words, is their action not on the glyoxalase but on the phenylglyoxal? It is worth noting that in every experiment on anti-glyoxalase reported in their original paper Dakin and Dudley [1913, 4] used phenylglyoxal as substrate and not methylglyoxal.

In the experiments described below some evidence is brought forward to show that pancreatic extracts do in some way change the phenylglyoxal. It cannot, of course, be said that they have no action on glyoxalase, and therefore that "antiglyoxalase" does not exist, until the action of pancreatic extracts has also been tried on methylglyoxal. It may be mentioned in passing, however, that it is difficult to imagine the significance and function of an anti-glyoxalase since it is not found in the blood stream and the removal of the pancreas does not increase the glyoxalase action of the tissues [Dakin and Dudley, 1913, 4].

Experimental details.

If by previous incubation with pancreatic extract the phenylglyoxal has undergone some change, it will not give rise to mandelic acid when subsequently added to a glyoxalase preparation even if all the antiglyoxalase factor is removed. This was the principle underlying the following experiments. After incubation overnight the mixture of phenylglyoxal and a pancreatic extract, which was always orange, was boiled to destroy any antiglyoxalase and then added to a glyoxalase solution prepared from chicken muscle in the usual way. In order to demonstrate that any change in the phenylglyoxal was not due to the boiling, the solution (0·2 g. in 10 cc.) was boiled before being added to the control flasks. It was shown repeatedly that the phenylglyoxal was in no way affected by boiling or even autoclaving (Exp. 7). There were always controls with and without pancreatic extract as shown in the following table:

Exp. 5. Pancreatic extract 20 cc. = 1 g.

Table VI.

No.	Extract added	Treated	Rotation	Notes
1	20 cc. saline	None	−3·02°	Controls
2	20 cc. saline	None	−2·91°	Controls
3	20 cc. water extract	Raw	+0·01	Orange colour
4	20 cc. water extract	Raw	+0·07°	and inhibition
5	20 cc. water extract	Incubated with the phenyl-	−0·23°	Orange colour
6	20 cc. water extract	glyoxal 24 hours and then boiled 13 mins.	−0·25°	and inhibition
7	20 cc. water extract	Boiled 13 mins.	−0·16°	Orange colour
8	20 cc. water extract	Boiled 13 mins.	−0·39°	and inhibition

There is therefore no mandelic acid formed from the phenylglyoxal, which was first treated with the pancreatic extract; but this is no evidence that the effect was due to destruction of the phenylglyoxal since the addition of boiled extract to the mixture containing glyoxalase also prevents the formation of mandelic acid. This means that either the phenylglyoxal was unchanged by the previous action of the pancreatic extract, which seems unlikely in view

of the marked change in colour and the development of precipitate, or that the "anti" factor, whether it acts on the phenylglyoxal or glyoxalase, is thermostable. Dakin and Dudley [1913, 4] found their antiglyoxalase preparations destroyed by heating to 85° for 10 minutes and this was confirmed in the case of a very weak antiglyoxalase solution (see Exp. 4).

It is generally agreed that any factor of an enzyme character would be destroyed by autoclaving even if protected by other substances, such as protein, or by high concentration. Exp. 5 was therefore repeated and all the various solutions were autoclaved at 120° for 20 minutes. It was necessary to show, first of all, that the phenylglyoxal itself was unaffected by autoclaving, and this was done by comparing the effect of glyoxalase on autoclaved and unautoclaved phenylglyoxal. A mixture of rat muscle and liver was used as the source of glyoxalase.

Table VII.

No.	20 % extract of rat liver and muscle	Phenyl-glyoxal 2 %	Treatment	Rotations
1	50 cc.	10 cc.	Control	−2·67°
2	50 cc.	10 cc.	Autoclaved	−2·95°

The phenylglyoxal solution can therefore be autoclaved with impunity.

Exp. 7. Chicken muscle as source of glyoxalase.

Table VIII.

No.	Extract added	Treatment	Rotation	Notes
1	10 cc. saline	None	−2·42°	Control
2	10 cc. saline	None	−2·40°	Control
3	10 cc. water extract	Incubated 24 hrs. with 0·2 g. phenylglyoxal and then the whole autoclaved	−0·09°	Inhibition. The mixture was bright orange before addition of muscle extract
4			−0·09°	
5	10 cc. water extract	None	−0·10°	Inhibition. Orange colour developed during incubation
6		None	−0·05°	
7	10 cc. water extract	Autoclaved before use	−0·92°	Some development of orange colour and partial inhibition
8		Autoclaved before use	−0·81°	

It is obvious that even autoclaving has not completely destroyed the inhibitory factor whatever it is. It is extremely improbable that anything of an "organised" nature would have survived such treatment. All trace of trypsin has gone, though it is difficult to destroy by boiling alone in such high concentration. It must be some thermostable substance present in the pancreatic extract which is producing the effect.

In order to make sure that the results observed were not due to differences of reaction, though this was very improbable in view of the excess of chalk used in every flask, the experiment was repeated exactly, except that every solution was neutralised to p_H 7·0 both before and after autoclaving. The result was the same as shown by Table VIII.

Exp. 8.

Table IX.

No.	Extract	Treatment	Rotation	Notes
1	10 cc. saline	—	−2·39°	Control
2	10 cc. water extract	Incubated overnight with phenylglyoxal and then autoclaved	−0·16	Inhibition
3	10 cc. water extract	Raw	−0·13°	Inhibition
4	10 cc. water extract	Autoclaved	−0·80°	Partial inhibition

Owing to the difficulty of removing the factor after its action on phenylglyoxal, these experiments do not definitely prove that its action is on the phenylglyoxal rather than the glyoxalase; but there are two facts which make this extremely probable. One is the development of the orange colour, which invariably accompanies inhibition, by the action of the pancreatic extract on the phenylglyoxal alone. The other is that the inhibition produced by the autoclaved extract on the whole mixture is only partial (as shown by Exp. 4, Nos. 7 and 8), while the inhibition produced by its action on the phenylglyoxal is complete.

Is methylglyoxal the precursor of lactic acid in muscle?

In the paper already referred to [Foster and Woodrow, 1924] the suggestion is made that certain facts of muscular metabolism, in particular the liberation of lactic acid at special surfaces where it is required for a special purpose in the series of phenomena that result in muscular movement, would be more easily explained if there were any evidence to show that the muscle has a specialised carbohydrate metabolism of its own; that is to say, if the sequence of chemical changes, which take place in the production of lactic acid from the carbohydrate stores of the muscle, were not necessarily the same as those taking place in other organs, where the lactic acid is merely a stage on the way to the complete combustion of glucose to carbon dioxide and water, with the liberation of all its available energy. In the muscle lactic acid is needed in relatively large quantities at a given surface at a given moment, and is produced as the result of a special stimulus, normally through a nerve. If there were this alternative course in the breakdown of glucose the mechanism controlling or inhibiting these changes would be different from that in an organ not so specialised; the liver for example. As already tentatively suggested such a differentiation would help to explain some of the anomalies of the diabetic condition.

It must be admitted that up to the present there is very little evidence in favour of this hypothesis, but one significant fact has accidentally come to light in the course of the work described in the earlier part of this paper. Some months ago the author (in collaboration with Mr C. E. Woodrow) made repeated attempts to test the pancreatic extract for the presence of antiglyoxalase in the way described above. Time after time the same difficulty occurred, and the experiments were useless because the controls were always negative. When a muscle extract prepared in the routine way was added to

phenylglyoxal no mandelic acid was obtained. This was repeated five or six times on different specimens of phenylglyoxal and no mandelic acid was ever produced. Later when another muscle extract (chicken) was used as the source of glyoxalase no further difficulty was experienced, and it was always possible to get an extract able to convert phenylglyoxal into mandelic acid. The reason for our lack of success in all the earlier experiments was that we had always used *rabbit* muscle. It is now clear, as the experiments described below prove, that rabbit *muscle* contains no glyoxalase.

It seemed impossible that the original discoverers of glyoxalase should not have found this exception to the general rule that, with the exception of the pancreas and certain specialised glands, all tissues contain glyoxalase, including the muscle of various animals, *e.g.* dog, fowl, cat, etc. An examination of their results [Dakin and Dudley, 1913, 1, 2, 3 and 4] shows that whenever the rabbit was the animal selected they used a mixture of muscle and *liver*. As the experiments described in detail below show, rabbit liver is very rich in glyoxalase, while from the muscle it is completely absent. This is a very significant fact.

Experimental results.

These experiments were all carried out in the routine way already described. In each case a 20 % extract of the muscle was used and the rotation is that obtained by the action of 50 cc. muscle extract on 0·2 g. phenylglyoxal. The final solution was always made up to 15 cc. and the reading taken in a 2 dm. polarimeter tube, mercury green being the source of light.

Table X.

Exp. no.	Source of glyoxalase	Rotation of mandelic acid	Notes
10	Rabbit muscle	−0·21°	No crystalline residue from the ether
	Rabbit muscle	−0·19°	
14	Rabbit liver	−2·50°	Typical crystals of mandelic acid
	Rabbit liver	−2·28°	
	Muscle from same animal	+0·44°	No crystalline residue
	Muscle from same animal	+0·39°	No crystalline residue
17	Rabbit liver	−2·17°	Mandelic acid crystals
	Rabbit liver	−2·26°	Mandelic acid crystals
	Muscle from same animal	+0·05°	No residue except traces of ammonium sulphate
	Muscle from same animal	+0·07°	

It is quite certain that rabbit muscle produces lactic acid in a perfectly normal way, in fact it is the muscle on which a great deal of experimental work has been done [Fletcher, 1911, 1913; Winfield and Hopkins, 1915]. The present author has frequently found that chopped rabbit muscle produces lactic acid in relatively large amount. The following figures will serve as examples. In each case the muscle was removed immediately after the death of the rabbit and rapidly minced. One fraction was at once ground in ice-cold alcohol and served as control, while the rest was left at room temperature for 24 hours in various buffered solutions and the lactic acid then estimated

in the routine way [Foster and Woodrow, 1924]. These figures are selected at random from a large number of experiments carried out for another purpose.

Table XI.

% of lactic acid in control	% of lactic acid after incubation	Notes
0·164 (1) 0·164 (2)	0·523 (1) 0·664 (2)	Incubated in a "Ringer" solution containing sodium bicarbonate (1) or borate (2)
0·16 0·13	0·44 0·47	Incubated in a "Ringer" solution containing bicarbonate
0·18 0·19	0·46 0·46	Incubated in a "Ringer" solution containing bicarbonate
0·12 0·14	0·54 0·53	Incubated in a "Ringer" solution containing bicarbonate
0·16	0·673	Incubated in a "Ringer" solution containing bicarbonate

It follows from these experiments that in rabbit muscle lactic acid is produced without the action of glyoxalase and, presumably therefore, it does not arise from methylglyoxal. On the other hand, the liver of the same animal is rich in glyoxalase and there is every reason to suppose, as Dakin and Dudley suggested [1913, 1914], that it plays an important part in the conversion of ketonic aldehydes into the corresponding α-hydroxy-acids, and that lactic acid is produced in this way in this organ from methylglyoxal. This is at present an isolated instance and the muscle of certain other animals, mammals as well as birds, has definitely been shown to contain glyoxalase, but it is possible that there are other animals like the rabbit which have no glyoxalase in their muscles. However, even one exception is important since it shows that methylglyoxal is not necessarily a precursor of lactic acid. It is significant that the amount of mandelic acid obtained by Dakin and Dudley [1913, 4] when muscle was used as the source of glyoxalase, was a good deal lower than that obtained from the same weight of liver. For example, for the dog the rotations observed were:

Liver	$-2{\cdot}3°$ to $-2{\cdot}58°$
Muscle	$-0{\cdot}45°$ to $-1{\cdot}13°$
Heart and tongue	$-0{\cdot}45°$

It is possible that where glyoxalase is found in muscle it serves to catalyse the breakdown of glucose into carbon dioxide and water, through methylglyoxal, in the general metabolism of the muscle, but plays no part in the special production of lactic acid as the result of a nerve stimulus, which results in contraction.

SUMMARY.

1. The factor found in the pancreas and able to inhibit the production of lactic acid by chopped muscle *in vitro* is not antiglyoxalase.

2. Some evidence is brought forward to show that the "antiglyoxalase" action is probably due to the action of some factor in the pancreas on phenylglyoxal rather than on the glyoxalase.

3. It is shown that rabbit muscle does not contain glyoxalase and the significance of this in the carbohydrate metabolism of muscle is discussed.

I wish to thank Dr Dudley for his interest and for the hospitality of his laboratory at one stage of this work, and Sir F. G. Hopkins for his continuous help and encouragement.

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CXVIII. A METHOD FOR THE ESTIMATION OF IODINE IN THYROID GLAND.

By FREDERICK ALFRED PICKWORTH.

From the Research Laboratories, Hollymoor, Northfield, Birmingham.

(*Received August 6th, 1925.*)

An examination of the published methods for the estimation of iodine in tissues has shown certain objections to their use for the special purpose of determining the iodine in thyroid gland.

Iodine is very reactive and is volatile so that its estimation is not easy. Many methods for its estimation have been put forward, the most important of which are briefly criticised below.

In comparing them however with the method to be given later, attention is drawn to the desirability of avoiding all processes involving unknown factors, and those subject to a large personal equation such as the colorimetric.

Baumann's method and its large number of modifications consist in the fusion of the gland with sodium hydroxide and potassium nitrate, followed by acidification and the extraction and estimation of the liberated iodine. The objection to all modifications of this method is that the effervescence which occurs on acidification causes an indefinite loss of iodine.

Hunter's method destroys the organic compounds by heating with a mixture of potassium and sodium carbonates and nitrates; the dissolved melt is treated while still alkaline with sodium hypochlorite; the solution is acidified and boiled to expel excess of chlorine, potassium iodide added and the iodine titrated with thiosulphate. The objections are the possible formation of oxychlorine compounds (Kendall) which would invalidate the result; and also the fact that the fusion process is inadequate with organic iodine compounds such as are met with in thyroid. Kelly and Husband also find that the process gives low results with seaweed.

Kelly and Husband's [1924] modification of Kendall's process was published while the present work was in progress. It consists of alkali fusion with sodium hydroxide and completion of the oxidation with potassium nitrate as in the Baumann method; acidification with phosphoric acid, and prolonged boiling to get rid of the nitrous acid. Sulphite is added and the SO_2 boiled off and then bromine is added, excess of which is also got rid of by prolonged boiling. Potassium iodide is then added and the liberated iodine is titrated.

One objection, as in the Baumann method, is the possible loss of iodine in the neutralisation of the mixture with acid, which liberates nitrous acid and much carbon dioxide.

Another objection is the fact, easily demonstrable in a test-tube, that bromides liberate iodine from potassium iodate in the presence of acid; an indefinite quantity of bromide is necessarily formed in the process of oxidation by the bromine.

The prolonged boiling and bromine vapour also present certain practical objections.

In Fellenberg's process [1924] the fusion process differs from the foregoing in that no potassium nitrate is added; but iodine is estimated colorimetrically. It is not intended by this author to be exactly quantitative since the results are multiplied by $\frac{5}{4}$ to allow for loss. It is a good method for the detection of minute amounts of iodine.

The method put forward depends upon the destruction of organic matter by fusion with alkali and oxidation of the resulting iodide to iodate with permanganate solution; excess of the latter is removed with animal charcoal, and after adding iodide the liberated iodine is titrated with thiosulphate.

The complete process is as follows:

0·25 g. of finely powdered dry thryoid (1) is weighed and introduced into a nickel crucible of 150 cc. capacity; 10 cc. 50 % sodium hydroxide (2) is added and mixed by rotating the crucible; it is covered with a lid having a hole $\frac{1}{8}''$ in the centre and heated slowly till the water is evaporated; it is next heated in an oven considerably below red heat but hot enough to give a clear fusion product in about an hour (3); the crucible is next heated over a large free flame protected so that the crucible and the lid can be rapidly raised to a dull red heat, which is maintained $\frac{1}{2}$—1 minute (4).

Allow the crucible to cool, add about 70 cc. water and place on a warm plate for about an hour, when gentle stirring will bring the whole into solution (5); transfer to a 250 cc. Erlenmeyer flask, add three drops 10 % sodium sulphite solution (6); acidify with 50 % sulphuric acid (7) from a burette, about 13 cc. being required (test acidity with a narrow strip of litmus paper mounted on a holder); add 3 cc. in excess, cool and add 5 cc. approx. $N/10$ permanganate; after 3 minutes add a suspension of charcoal (8); when decolorisation is complete filter through a suction filter (9) washing with a little water, or through a pleated filter paper (10); add a crystal of potassium iodide and titrate immediately with freshly prepared $N/100$ thiosulphate, adding starch only towards the end of the titration.

Each 1 cc. thiosulphate $\times$ 85 gives the milligrams of iodine in 100 g. of dried gland when 0·25 g. is taken for the analysis. It is found convenient to work with batches of six thyroids.

NOTES ON THE METHOD.

(1) The thyroid should pass a 60 sieve.
(2) The soda should be free from nitrate and from chloride, the commercial "Soda by alcohol" answers well.
(3) Potassium nitrate must not be added.
(4) Prolonged heating at red heat causes a slight loss.

(5) A few carbon particles remain which do not affect the result. See Exp. 7 below in which a small amount of charcoal is added at the initial stage.

(6) This does not apparently affect the result but is added as a precaution against the possibility of iodine being liberated at this stage by traces of impurity such as iron.

(7) The sulphuric acid must be chemically pure.

(8) Shake 20 g. animal charcoal with 1000 cc. water and 2 cc. sulphuric acid. The British Drug Houses "Animal charcoal purified by acid" is satisfactory. Use a pipette with a very wide jet for measuring rapidly before sedimentation takes place. The amount required is determined by that which will reduce 5 cc. acidified $N/10$ permanganate in not less than 5 or more than 20 minutes: this is usually 6 cc.

(9) The filtrate must be inspected very carefully to see that it is absolutely free from colour and from minute particles of carbon.

(10) If a pleated filter is used it is found more convenient, instead of washing, to weigh against another paper as a counterpoise and make the small correction for the amount of fluid on the paper and in the flask, *e.g.* if the weight on the paper is 3 g. and that in the flask 0·5 g. the corrected figure will be 113·5/110 with an amount of fluid of 110 cc.

Suitable suction filters are the Jena Sintered Glass 17G 4/< 7 filters.

The permanganate may be added to the alkaline solution instead of after acidification if desired, in which case addition of sulphite is unnecessary.

Iron should be removed from the solution if the thyroids contain a large excess of blood by adding to the solution after acidification enough soda to make just alkaline and after standing some hours filtering off the precipitated iron, acidifying, adding 3 cc. of 50 % acid and proceeding as above.

Test experiments with experimental mixtures.

Solutions used. Potassium iodide $N/200$. Thiosulphate $N/100$ made fresh each day by diluting 10 cc. of $N/10$ to 100 cc. The $N/10$ thiosulphate was preserved with a crystal of thymol and 25 cc. were equivalent to 0·1226 g. $K_2Cr_2O_7$.

In tests 1–11, 1 cc. of $N/200$ KI was measured by means of a 1 cc. D and R pipette the standardisation of which was checked by weighing. The theoretical titration figure in these experiments (1–11) was therefore 3·00 cc.

Exps. 1–9 consisted of the latter half of the process; *i.e.* without addition of alkali and the fusion process. Variations in amount of reagents and other factors were introduced into the test processes below to show the flexibility of the process in allowing such variation:

(1) *The amount of acid* (instead of 3 cc.) was varied as follows:

	Acid (cc.)	Titre
	0·5	2·60
	1·0	2·86
	2·0	3·02
	4·0	2·99
	5·0	3·03
Blank	12·0	0·00

The amount of acid must therefore be at least 2 cc.

(2) *The amount of permanganate* (instead of 5 cc.) was varied as follows:

$KMnO_4$ (cc.)	Titre
0·5	2·93
1·0	3·02
2·0	2·97
5·0	3·01
10·0	2·97

The amount may vary considerably.

(3) *Temperature of action of* $KMnO_4$:

	Titre
Ordinary	3·01
Hot 60—70°	2·95
Hot 60—70°	2·97

The temperature is not important.

(4) *Time of action of* $KMnO_4$:

	Titre
Immediate	2·99
1 min.	3·02
2 mins.	3·04
5 „	3·07
90 „	3·07
90 „	3·06

The permanganate oxidises the iodide immediately.

(5) *The amount of charcoal* (instead of 6 cc.) was varied as follows:

cc.	Titre
5	3·05
7	3·02
9	3·00

The amount of charcoal may vary within reasonable limits.

(6) *The amount of iodine*:

cc.	Titre
1	3·01
2	5·97

Also see No. 10.

(7) *Charcoal* (0·5 cc. suspension) *present at start*:

Titre 3·03

A small quantity of carbon present in the process will not affect the result.

(8) *Time in contact with the charcoal after the decoloration is completed*:

mins.	Titre
30	2·97
120	2·98

The charcoal does not reduce the iodate.

(9) *Effect of salts*:

	Titre
10 cc. NaOH neutralised, 2·5 cc. excess acid	3·01
10 cc. NaOH neutralised, 2·5 cc. excess acid (blank)	0·00
10 cc. NaOH neutralised after previously adding 5 drops of 10 % sulphite...	3·02

The salts present in the actual experiment do not affect the result.

(10) The following were experimental mixtures of the iodide solution and 0·25 g. of meat mixed and treated by the complete process as above:

cc.	Titre
1	2·96
2	5·93

The amount of iodine may vary widely.

(11) *Temperature and amount of heating varied*:

30 mins. slow heating till clear, then as follows:	Titre
1. Melt, not red heat	2·90
2. Red hot 1 min., quick heating	3·00
3. Red hot 3 mins.	2·98
4. Red hot 10 mins.	2·91
9. Blank with starch	0·01
10. Blank with meat	0·04

The amount of heating is important. Also see Exp. 12.

(12) *Thyroid gland*:

	Titre
Just red hot	1·65
Red hot 1 min.	1·62
Red hot 5 mins.	1·62
Red hot 10 mins.	1·61
Red hot 30 mins.	1·61
Well heated but always under red heat	1·64

There is a slight loss by prolonged heating at red heat.

The above show that there is considerable liberty allowed in the amount of reagents and other factors of the method.

Duplicate experiments give results which are correct to one drop of titrating solution. All duplicate results were obtained at different times.

Over 100 human thyroid glands have been analysed by the method and results obtained ranging from 5–520 mg. per 100 g. of dried tissue; and 0·4–52·5 mg. per whole gland obtained, details of which are being published in the *Journal of Mental Science*.

The above work was carried out in the laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham.

My best thanks are due to Sir Frederick Mott, K.B.E., M.D., F.R.S., for suggesting this work.

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CXIX. THE SEXUAL GLANDS AND METABOLISM.

IV. THE INFLUENCE OF INJECTIONS OF EMULSIONS OF TESTES AND PROSTATE AND OF INSULIN-LIKE TESTICULAR EXTRACTS UPON THE NITROGEN METABOLISM OF NORMAL, CASTRATED AND THYROIDECTOMISED RABBITS.

By VLADIMIR KORENCHEVSKY and MARJORIE CARR.

From the Lister Institute, London.

(*Received August 12th, 1925.*)

I. Historical.

Castration produces a fall in the nitrogen metabolism of most dogs and rabbits [Korenchevsky, 1925, 1]. Dog's testes contain specific hormones which probably prevail when injected in the form of testicular emulsion [Korenchevsky, 1925, 2]. This is indicated by an increase in the nitrogen metabolism of most dogs after these injections. Thyroidectomy in dogs does not change this effect. It seems that in dogs the sexual hormones do not produce their influence upon nitrogen metabolism by means of the thyroid or at any rate not exclusively. On the other hand, Korenchevsky and Carr [1925] found that homologous testis emulsion when injected subcutaneously into rabbits slightly decreased the nitrogen metabolism during the period of injections and in most cases during the succeeding 5–9 days. This effect of testis emulsion cannot, however, be considered to be a specific one because of the similar effect of kidney emulsion.

An emulsion of prostate, injected alone or in combination with testis, increased the nitrogen metabolism up to 11 %. The rise in N output was manifested more particularly during the 2 or 3 days following the period of injections.

The diminished N metabolism after the injection of testis emulsion in the rabbit was contrary to expectation because castration also produces a fall in the nitrogen metabolism [Korenchevsky, 1925, 1]. Therefore we were inclined to attribute these contradictory results chiefly to the possible presence in the rabbit's testis of two different principles, one increasing, the other decreasing nitrogen metabolism, the latter prevailing when an emulsion of this organ is injected. Ashby [1923], Baker, Dickens and Dodds [1924],

Ivy and Fisher [1924] and Best, Smith and Scott [1924] have found that various tissues, including kidney, contain an insulin-like substance. And according to Fitz, Murphy and Grant [1922], Wilder, Boothby, Barborka, Kitchen and Adams [1922], MacLeod and Allan [1923] and Kudrjavzeva [1925] injections of insulin lower the nitrogen metabolism. Therefore, if testes contain an insulin-like factor, this latter substance may partly be the cause of the decreased nitrogen metabolism.

The experiments to which we shall refer in this paper seem to justify this theory.

II. The present investigation.

Technique.

Twenty-three experiments were performed on 12 rabbits, of which 4 were castrated (Nos. 14, 22, 26, 33), 4 thyroidectomised (Nos. 24, 31, 38, 39), 3 both thyroidectomised and castrated (Nos. 23, 17, 40) and 1 normal (No. 41). The technique of the metabolism experiments, the preparation of the emulsions of prostate and testis and the amounts injected are the same as those described in a previous paper [Korenchevsky and Carr, 1925].

Mr F. H. Carr at the laboratories of the British Drug Houses isolated insulin-like substances from bull's testes and kindly supplied us with the amount necessary for our experiments—we wish to thank him and his firm for this as well as for the following data concerning the method of purification and the influence of these substances upon the blood-sugar of rabbits, the latter being determined by Dr S. W. Underhill.

The method of isolation briefly consisted in the precipitation of alcoholic extracts of bull's testes at different p_H values, namely, p_H 6·8 (testicular extract A), p_H 5·0 (testicular extract B) and p_H 4·0 (testicular extract C). These three substances were converted to hydrochlorides by Dudley's method. They reduced the blood-sugar content of rabbits as follows:

Testicular extract A.	2·5	mg.	per	kg.	reduced	the	blood-sugar	to	55 mg. per 100 cc.
	4·0	,,	,,	,,	,,	74	,,	,,	
Testicular extract B.	0·15	,,	,,	,,	,,	44	,,	,,	
	0·25	,,	,,	,,	,,	50	,,	,,	
	0·4	,,	,,	,,	,,	51	,,	,,	
Testicular extract C.	2·0	,,	,,	,,	,,	45	,,	,,	
	3·0	,,	,,	,,	,,	54	,,	,,	

For our experiments these three fractions were each dissolved in sterile distilled water and injected subcutaneously in amounts varying between 0·8 mg.–2·3 mg. per diem and per kg. of the rabbit's weight. The amounts injected per diem and kg. are given in the tables. Each experiment was divided into four periods, namely, the first control period of 3 days before the injections, the period of injections lasting 3 days, followed by two or three control periods, each of which usually lasted 3 or sometimes one of them only 2 days. When the doses were increased and we feared convulsions might follow, the injection period only lasted for 1 day. For this same reason in

Exps. 2, 7, 12, 15 and 16 the rabbits were injected with small doses (about 0·8 mg. per kg.) for 3 days. Five days after the injection period, when the nitrogen metabolism of the thyroidectomised animals was still below normal, another larger injection was given once only.

In normal or castrated animals fractions A, B and C were injected in the same or larger amounts.

In order not to increase the amount of tabular matter, firstly, we do not include the experiments on testicular extract A, because its activity was small, nor the experiments on the injections of a mixture of A, B and C extracts, because the results obtained were nearly identical with those with B extract. Secondly, though the nitrogen intake in the food and output in the urine and faeces were estimated carefully as in our previous work, they are not included in the tables. The daily intake of nitrogen was always the same during a whole experiment. It only varied slightly in different experiments, because the cabbage could be stored for the length of one experiment only (about 18 days), but not for all the experiments on the same animal [for further details *see* Korenchevsky and Carr, 1925, pp. 77, 78; Korenchevsky, 1925, 3].

Description of the Experiment.

The actual figures and details of the experiments are given in Tables I and II and the results obtained are summarised in Tables III and IV. In the latter the figures show the average percentage change above (+) or below (−) normal in the urinary nitrogen excretion and in the urinary flow during the period of injections and the two or three following control periods, compared with that of the same animal during the first control period before the injections. In Tables III and IV the average percentage changes in thyroidectomised animals are compared with those in normal or castrated animals with thyroids. The average figures of the urinary nitrogen and flow of the latter animals were taken from experiments published previously [Korenchevsky and Carr, 1925]. As was mentioned above, the experimental technique was exactly the same in both.

Injections of Testis Emulsion.

The details of these experiments on rabbits 23 and 24 are given in Table I (Exps. 1 and 3) and Table II (Exp. 10). Before the period of injections in Exp. 1 the nitrogen balance was positive, but was negative in this period in Exps. 3 and 10. The results of all three experiments were almost identical, namely, a quite definite decrease in the nitrogen excreted in the urine (15·5, 14·5 and 7·2 % respectively below normal) and an increase in the nitrogen balance. This was always most pronounced, and in two experiments was only present in the control period following the injections.

A comparison of the results obtained on animals with thyroids and on those without thyroids (see Table III) shows that the injection of testis

Table I. *The influence of subcutaneous injections of insulin-like extracts from testis and of emulsions of testis and of prostate upon the nitrogen metabolism of rabbits.*

No. of experiment and rabbit	No. of period	Weight of rabbit g.	Volume of urine excreted cc.	Nitrogen in urine g.	Balance of nitrogen g.	Remarks
Exp. 1						
Rabbit 23 (thyroidectomised and castrated)	I	3575	448	9·43	+0·11	I control period
	II	3577	462	9·57	−0·13	Injection of testis emulsion
	III	3605	375	8·27	+1·17	II control period
	IV	3640	348	8·81	+0·63	III control period
	V	3655	504	8·69	+0·78	IV control period
Exp. 2						
Rabbit 23	VI	3490	651	8·07	−1·56	I control period
	VII	3485	628	7·56	−1·05	Injection of 0·9 mg. testis extract C
	VIII	3480	571	7·60	−1·09	II control period
	IX	3480	562	7·48	−0·97	III control period
	X	3500	518	7·17	−0·66	Injection of 1·7 mg. testis extract C
	XI	3475	777	7·83	−1·32	IV control period
Exp. 3						
Rabbit 23	XII	3420	611	7·58	−1·11	I control period
	XIII	3403	565	7·85	−1·38	Injection of testis emulsion
	XIV	3420	420	6·48	−0·01	II control period
	XV	3420	502	6·93	−0·46	III control period
Exp. 4						
Rabbit 38 (thyroidectomised)	I	2695	418	6·50	−0·52	I control period
	II	2695	385	6·72	−0·74	Injection of prostate and testis emulsion
	III	2685	371	6·51	−0·53	II control period
	IV	2680	373	6·72	−0·74	IV control period
Exp. 5						
Rabbit 40 (thyroidectomised and castrated)	I	2560	424	6·25	−0·46	I control period
	II	2565	434	6·43	−0·64	Injection of prostate emulsion
	III	2545	485	6·24	−0·45	II control period
	IV	2545	428	6·14	−0·35	III control period
Exp. 6						
Rabbit 17 (thyroidectomised and castrated)	I	2590	488	8·71	−0·54	I control period
	II	2627	286	7·98	+0·19	Injection of emulsion of prostate and testis
	III	2620	518	8·23	−0·06	II control period
	IV	2620	441	8·57	−0·40	III control period
	V	2595	434	8·56	−0·39	IV control period
Exp. 7						
Rabbit 17	VI	2580	390	7·36	−0·14	I control period
	VII	2550	462	7·34	−0·12	Injection of 0·8 mg. of testis extract B
	VIII	2565	329	6·99	+0·23	II control period
	IX	2560	392	6·93	+0·29	III control period
	X	2520	616	6·92	+0·30	Injection of 1 mg. of testis extract B
	XI	2545	273	6·16	+1·06	IV control period
Exp. 8						
Rabbit 17	XII	2550	401	7·90	−0·15	I control period
	XIII	2580	380	7·80	−0·05	Injection of emulsion of prostate and testis
	XIV	2585	343	7·57	+0·18	II control period
	XV	2565	406	7·70	+0·05	III control period

TE. In this and the following tables figures showing N intake and N content of faeces are omitted for brevity's sake. The figures are calculated for 1 week so as to be comparable. The figures for insulin-like extracts represent the amount injected per diem and kg.

Table II. *The influence of subcutaneous injections of insulin-like extracts from testis and of emulsions of prostate and of testis upon the nitrogen metabolism of rabbits.*

No. of experiment and of rabbit	No. of period	Weight of rabbit g.	Volume of urine excreted cc.	Nitrogen in urine g.	Balance of nitrogen g.	Remarks
Exp. 9						
Rabbit 24 (thyroidectomised)	I	3185	530	8·49	+0·62	I control period
	II	3215	560	8·37	+0·74	Injection of prostate emulsion
	III	3168	601	8·52	+0·59	II control period
	IV	3195	478	8·71	+0·40	III control period
	V	3275	411	8·36	+0·75	IV control period
Exp. 10						
Rabbit 24	XII	3495	527	9·32	−0·92	I control period
	XIII	3485	548	8·92	−0·52	Injection of testis emulsion
	XIV	3495	462	8·65	−0·25	II control period
	XV	3485	586	8·82	−0·42	III control period
Exp. 11						
Rabbit 31 (thyroidectomised)	I	2555	537	7·65	−0·29	I control period
	II *b*	2567	392	5·70	+1·66	Injection of 1·4 mg. testis extract B
	V	2565	553	7·51	−0·15	IV control period
Exp. 12						
Rabbit 31	VI	2542	504	7·25	−1·93	I control period
	VII	2505	537	7·25	−1·93	Injection of 0·8 mg. testis extract B
	VIII	2520	392	6·36	−1·04	II control period
	IX	2545	436	6·28	−0·96	III control period
	X	2550	392	5·73	−0·41	Injection of 1·0 mg. testis extract B
Exp. 13						
Rabbit 31	XII	2408	488	7·33	−1·41	I control period
	XIII	2370	567	7·34	−1·42	Injection of prostate emulsion
	XIV	2330	553	7·25	−1·33	II control period
	XV	2320	495	7·15	−1·23	III control period
Exp. 14						
Rabbit 33 (castrated)	I	2570	586	8·72	−0·18	I control period
	II	2555	525	8·48	+0·06	Injection of 1·4 mg. testis extract C
	III	2560	478	9·07	−0·53	II control period
	IV	2580	455	8·76	−0·22	III control period
	V	2565	436	8·41	+0·13	IV control period
Exp. 15						
Rabbit 33	VI	2627	495	8·28	−0·52	I control period
	VII	2620	490	7·80	−0·04	Injection of 1·2 mg. testis extract C
	VIII	2640	403	7·91	−0·15	II control period
	IX	2640	488	8·00	−0·24	III control period
	X	2650	392	8·06	−0·30	Injection of 2·3 mg. testis extract C
	XI	2645	455	7·80	−0·04	IV control period
Exp. 16						
Rabbit 14 (castrated)	I	3285	455	8·67	+0·39	I control period
	II	3295	420	8·40	+0·66	Injection of 0·9 mg. testis extract B
	III	3310	378	8·52	+0·54	II control period
	IV	3310	413	8·30	+0·76	III control period
	V	3300	546	8·30	+0·76	Injection of 1·3 mg. testis extract B
	VI	3305	378	8·19	+0·87	IV control period
Exp. 17						
Rabbit 26 (castrated)	I	2197	432	7·10	0	I control period
	II	2135	487	7·11	−0·01	Injection of 1·3 mg. testis extract B
	III	2160	348	7·66	−0·56	II control period
	IV	2160	387	7·10	0	III control period
	V	2165	395	6·98	+0·12	IV control period
Exp. 18						
Rabbit 41 (normal)	I	2880	443	9·04	−0·23	I control period
	II	2895	364	8·29	+0·52	Injection of 1·5 mg. testis extract B
	III	2890	280	9·03	−0·22	II control period

Table III. *Average percentage change from the normal in the N output in the urine of rabbits after injection of insulin-like extracts from testis and of different emulsions.*

Injection	Number of experiments averaged	Thyroid present (+) or removed (−)	Experimental periods: Injection period 3 days	II control period 2 days	III control period 3 days	IV control period 2 days
Kidney emulsion	3	+	− 4·4	− 4·9	−4·8	.
Testis emulsion	4	+	− 4·6	− 2·5	−2·4	.
	3	−	+ 0·2	−12·4	−7·1	−7·9
Prostate emulsion	4	+	+ 3·1	+ 9·0	+1·5	+3·3
	3	−	+ 0·5	− 0·2	−0·5	−1·6
Prostate and testis emulsion	5	+	+ 0·5	+ 9·2	+3·3	+1·3
	3	−	− 2·1	− 3·3	−0·3	−1·7
Testis insulin B per diem and kg.						
0·9 mg.	1	+	− 3·2	− 1·8	−4·3	.
0·8	2	−	0	− 8·6	−0·7	.
1·3–1·5	3	+	− 4·2	− 0·8	.	.
1–1·4	3	−	−17·5	−26·9	.	.
Testis insulin C per diem and kg.						
1·2–2·3 mg.	3	+	− 3·9	− 2·0	−1·7	.
0·9–1·7	2	−	− 8·8	− 4·7	−7·4	.

Table IV. *Average percentage change from the normal in urinary flow of rabbits after injection of insulin-like extracts from testis and of different emulsions.*

Injection	Thyroid present (+) or removed (−)	Injection period	II control period	III control period
Kidney emulsion	+	− 5·5	− 4·6	+ 5·0
Testis emulsion	+	− 8·8	−15·8	−12·0
	−	− 0·2	−19·9	− 9·6
Prostate emulsion	+	− 5·1	− 0·1	− 8·6
	−	+ 8·1	+13·7	− 2·4
Prostate and testis emulsion	+	− 2·4	−12·7	−13·3
	−	−18·2	− 6·6	− 6·4
Testis insulin B	+	+ 1·8	−22·5	− 9·8
	−	+ 1·5	−17·9	− 6·8
Testis insulin C	+	−10·4	−15·0	−12·4
	−	−12·0	−15·9	−13·7

emulsion decreased the nitrogen metabolism of thyroidectomised rabbits much more than that of normal or castrated animals.

Urinary flow was also decreased in the period following the injections, but, as the corresponding figures in Table IV show, there was no essential difference in this respect between the animals with and those without thyroids. This decrease in flow is incapable of interpretation, because we have not measured the water expired nor the water content of the faeces. Moreover, only in one experiment (No. 1) of the three was there an increase in weight. Therefore, there are no definite indications that the water is retained in the organism. The percentage of nitrogen excreted in the dried faeces was slightly increased in two experiments (Nos. 3 and 10) and slightly decreased in one (No. 1).

Injections of Prostate Emulsion.

The details of these experiments on rabbits Nos. 24, 31 and 40 are given in Table I (Exp. 5) and Table II (Exps. 9 and 13). These figures and a comparison of the results obtained on rabbits with thyroids with those on rabbits without thyroids show that the increase in nitrogen metabolism produced by the injection of prostate emulsion in animals with thyroids was not observed in the thyroidectomised animals.

Table IV shows that the excretion of urine during the injections of prostate emulsion and the succeeding period was increased in all thyroidectomised animals, whereas that of rabbits with thyroids was on an average slightly decreased.

There were no constant changes in the percentage of nitrogen excreted in the faeces as a result of the prostate injections.

Injections of a Mixed Emulsion of Testes and Prostate.

The details of these experiments on rabbits Nos. 17 and 38 are shown in Table I (Exps. 4, 6 and 8). The results of these experiments agree well with those of the two previous groups as is shown clearly in Table III.

The increase in nitrogen metabolism produced in rabbits with thyroids after the injection of prostate alone or of prostate and testis simultaneously was not found in the thyroidectomised rabbits. Moreover, when an emulsion of both prostate and testis was injected into the latter animals, on an average, a slight decrease was observed in the nitrogen metabolism, *i.e.* a change typical of testicular emulsion.

Therefore these groups of experiments show, firstly, that in order to produce an increase in the nitrogen metabolism after injection of prostate emulsion the thyroid gland must be present; secondly, that in thyroidectomised animals, when an emulsion of both testis and prostate is injected, the influence of the former gland prevails. The latter result is also corroborated by the decreased urinary flow in Exps. 4, 6 and 8, *i.e.* a change in the excretion of urine typical of the influence of testicular and not of prostate emulsion was observed. No definite or constant change was obtained in the percentage of nitrogen excreted in the faeces.

A slight increase in weight of rabbit No. 17 was observed during the injection period and the following control periods, but rabbit No. 38 slightly decreased in weight in the control periods following the injections.

Injections of Insulin-like Extracts (B *and* C) *of Bull's Testes.*

Extract B was injected into rabbits Nos. 14, 17, 26, 31 and 41 and details are given in Table I (Exp. 7) and Table II (Exps. 11, 12, 16, 17 and 18). Extract C was injected into rabbits 23 and 33 (see Table I (Exp. 2) and Table II (Exps. 14 and 15)). The difference between the influence produced by extracts B and C upon nitrogen metabolism was only of a quantitative nature and was

due to the presence of a greater amount of testicular insulin in extract B than in C (see the influence of these extracts upon the blood-sugar of rabbits mentioned above). When 0·8–1·5 mg. of extract B or 1·2–2·3 mg. of extract C per kg. and diem were injected subcutaneously into normal or castrated rabbits, in nearly all cases, only a slight fall was produced in the nitrogen metabolism. Similar or even smaller doses injected into thyroidectomised rabbits produced a much more pronounced decrease in the nitrogen metabolism (see Table III). This effect lasted and, in some cases, was only present or most pronounced in the control periods following the injections.

The percentage of nitrogen in the faeces was slightly increased in about half of the experiments and was decreased or not altered in the other half.

Urinary flow was quite definitely decreased in all cases during the control periods (5–8 days) following the injections, but during the injection period itself it was increased in five cases (36 %) and decreased in nine cases (64 %).

The weights of the animals under the influence of the injections changed a little in both directions; for example, during the control period immediately following the injections half increased and half decreased in weight. Therefore it was quite impossible to establish any relationship between the change in weight and the change in urinary flow, which, as we mentioned above, was diminished in all animals during this same period.

Discussion.

The presence or absence of the thyroid gland seems to be of importance for the changes in the nitrogen metabolism produced in rabbits by emulsions or extracts of testis and prostate glands. Testis emulsion and insulin-like substances obtained from the testis produced only a slight fall in the nitrogen metabolism of normal or castrated animals, probably because the thyroid hormones counteract this influence. In a previous paper [Korenchevsky, 1914] it was shown definitely that the thyroid gland is a great stimulant to nitrogen metabolism. Therefore it is quite reasonable to suppose that, as this effect was lacking in the thyroidectomised animals, the fall in the nitrogen metabolism after injections of testis emulsion and testicular insulin-like substances would be much greater than in the animals with thyroids. These observations agree well with the experiments of Bodansky [1923], Houssay and Busso [1924] and Ducheneau [1924], who found that toxic effects and lowering of blood-sugar produced by insulin were more pronounced in thyroidectomised than in normal animals.

The similarity between the influence of testis emulsion and that of insulin upon nitrogen metabolism and to some extent upon the excretion of urine suggests that the fall in nitrogen metabolism and urinary flow obtained in rabbits after the injection of testis emulsion might be partly due to the presence of insulin-like substances in the testes. The same explanation might apply to the decrease in the nitrogen metabolism of rabbits after the injection of kidney emulsion [Korenchevsky and Carr, 1925].

Experiments with prostate emulsion injected into normal and thyroidectomised rabbits have shown that the thyroid gland must be present in order to obtain an increase in the nitrogen metabolism: in thyroidectomised rabbits an emulsion of prostate alone produced practically no change in the nitrogen metabolism, whereas an emulsion of both prostate and testis usually decreased the nitrogen metabolism, although to a less degree than when an emulsion of testis alone was injected.

It seems probable, therefore, that the prostate emulsion produced its influence upon the nitrogen metabolism of rabbits indirectly through the thyroid gland. This mechanism is not found to work with the testicular emulsion, which, as the experiments on dogs show [Korenchevsky, 1925, 2], increases the nitrogen metabolism of thyroidectomised as well as that of normal and castrated animals.

Unfortunately it is not possible from our experiments to explain the mechanism which decreases urinary flow after the injections of testis emulsion and testicular insulin-like substances.

In our experiments thyroidectomy did not influence the changes in the excretion of urine produced by testis emulsion or insulin-like substances, but it did affect the change in flow after injections of prostate emulsion.

Conclusions.

1. An homologous emulsion of testis decreased the nitrogen metabolism of thyroidectomised rabbits much more than that of normal or castrated animals.

2. This emulsion also decreased urinary flow to about the same degree in rabbits both with and without thyroid glands.

3. The insulin-like substances isolated from testes and injected into thyroidectomised rabbits also produced a more pronounced decrease in nitrogen metabolism than in rabbits with thyroids.

4. These substances decreased the excretion of urine in both thyroidectomised and non-thyroidectomised animals during the periods following the period of injection, as did testicular emulsion. During the period of injection itself the flow of urine increased in about one-third of the experiments and decreased in the other two-thirds.

5. Thyroid hormones appear to counterbalance the depressing influence on N metabolism of rabbits produced by an emulsion of rabbit's testis or of testicular insulin-like substances.

6. The similarity between the effects of testicular emulsion and of the insulin-like substances extracted from testis suggests that in the former case the fall in nitrogen metabolism, and possibly in urinary flow, is in part due to the presence of insulin-like substances in testes.

7. The same explanation might apply to the decrease in the nitrogen metabolism of rabbits after an injection of kidney emulsion since insulin-like substances have also been isolated from kidney.

8. An homologous emulsion of prostate alone produced practically no change in the nitrogen metabolism of thyroidectomised rabbits, whereas in that of rabbits with thyroid glands a definite increase was observed.

9. This latter increase was also absent when an homologous emulsion of both prostate and testis was injected into thyroidectomised rabbits; instead, on an average, a slight decrease was observed, indicating the predominance of the testicular part of this mixed emulsion.

10. Prostate emulsion appears to increase nitrogen metabolism of rabbits by stimulating the activity of the thyroid gland.

11. Prostate emulsion increased the flow of urine in the thyroidectomised rabbits, whereas, on an average, the excretion of urine was decreased in rabbits with thyroid glands.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We also wish to express our sincere gratitude to Prof. C. J. Martin for his continuous support and criticism of this work.

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CXX. A LABORATORY APPARATUS FOR THE WET GRINDING OF PLANT TISSUES OUT OF CONTACT WITH AIR.

By WILLIAM ARTHUR ROACH.

From the Department of Insecticides and Fungicides, Rothamsted Experimental Station, Harpenden.

(*Received August 22nd, 1925.*)

In extracting the proteins from potato tubers great difficulty is experienced on account of the action of oxygen on tyrosine, causing in the early stages of the experiment the formation of melanin, the removal of which is difficult once it has been formed. An apparatus has therefore been devised in which the tubers may be finely pulped and the pulp filtered in the absence of oxygen, the exclusion of the latter being sufficiently thorough for the filtered juice to be of only a faint blue colour[1] instead of the intense black colour resulting when oxygen is present.

Since the yield of protein is likely to be proportional to the number of cells of which the walls are broken, a high degree of efficiency of grinding was also desirable. It was secured by means of a rapidly rotating alundum wheel tearing apart the tissues of the tuber. Turgid potato tubers have, in fact, been pulped with the machine at a rate of about 1 kg. per hour sufficiently finely to rupture almost every cell wall, without, however, breaking the starch granules.

Since the machine seems capable of application to many biochemical problems a brief description and diagram are given below in the hope that it may be of use to other workers.

An inverted glass bell-jar *A*, which serves as a receiver for the pulp, is suspended by its rim through a circular hole in a table *B*, a ring of rubber, *C*, acting as a cushion between the two. The bell-jar is held securely in position by the brass plate *D*, which is firmly attached to the table by means of three bolts and thumb screws *F* (one only shown in the diagram). A second ring of rubber *E*, placed between the brass plate and the bell-jar, serves to make an air-tight joint between them. Passing through the centre of the plate *D*, is a shaft and bearing *H*, which carry the alundum grinding wheel, *G*, on the lower end, and at the upper end can be connected by a leather band to a $\frac{1}{4}$ H.P. motor.

[1] This blue colour forms the subject of a note published in the *Annals of Botany*.

The potatoes to be ground up are inserted through a cylindrical brass tube *I*, which is fitted tightly into a hole in the brass plate *D* by means of screws, so that its basal end almost touches the grinding wheel. The tube is made in sections which may be screwed together so that its length may be altered at will. A second but much shorter brass tube of the same diameter, *J*, is soldered into a second hole in the plate lying just to the side of the first: this, for the sake of convenience in drawing, is shown out of position in the diagram. A piece of strong rubber tubing *K* (the inner tube of a small motor-bicycle is of a convenient size and curvature for the purpose) is attached to the brass tube *J*, and is closed at its free end with a block of wood *L*, into which

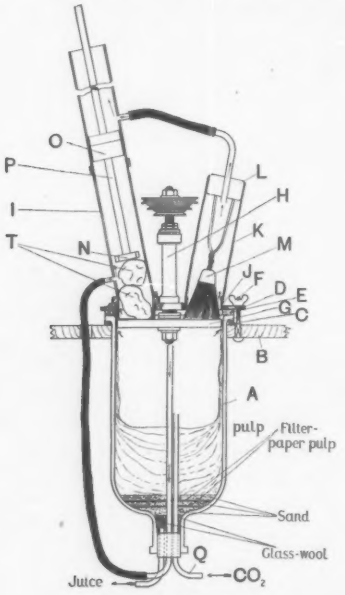

Fig. 1.

is fitted the handle of a steel wire brush *M*. The rubber tube is sufficiently long for the brush, in a position of rest, to be just free of the grinding wheel, but it allows sufficient freedom of movement to permit the brush to be brought down on the surface of the rotating wheel to clean it without either discontinuing the grinding or risking the introduction of oxygen. The three parts of the apparatus are connected up together, by means of glass and rubber as shown in the diagram, in such a way that the whole of the air in the apparatus can be replaced by an inert gas, not lighter than air, which enters under slight pressure by the inlet tube *Q*, passes in the directions indicated in the diagram by arrows, and finally emerges from the top of the tube *I*. In the case under consideration carbon dioxide was used to displace the air, though it is probable

that nitrogen might have served equally well. During an experiment a continuous stream of gas is kept passing through the apparatus in order to displace any air that may be carried in when the potatoes are introduced into the tube *I*. The tubers are lowered slowly, one at a time, to the base of the tube *I* on the end of a long skewer with a corkscrew end, which may be disengaged from the potato by unscrewing. Not more than two tubers at a time are put in the tube, otherwise there is a risk of jamming. They are pressed

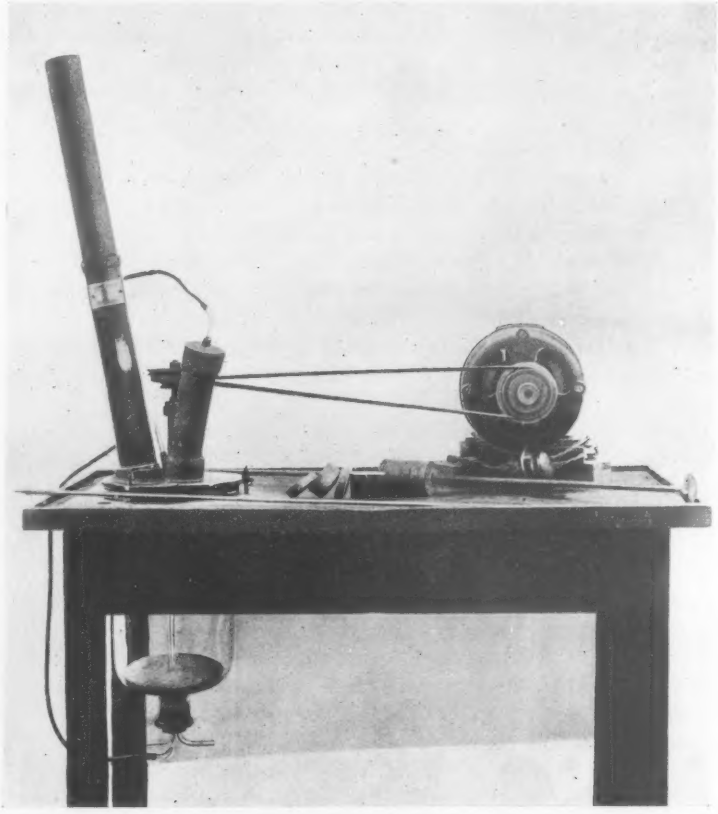

Fig. 2.

down against the grinding wheel by means of a plunger, the end *N* of which is about half the area of the cross section of the tube; this is sufficiently large to prevent the slipping of the end past a tuber and small enough to cause no serious introduction of oxygen when the plunger is pushed into the tube. A cylindrical piece of lead *O*, is screwed on to the rod *P*, of the plunger, about

half-way up its length to weight it: additional perforated leaden cylinders may be slipped over the rod to increase the pressure on the tubers. The wheel is spun at about 1500 revolutions per minute by means of a $\frac{1}{4}$ H.P. electric motor. The resulting pulp is quite white and is sufficiently liquid to run down the sides of the bell-jar, if turgid tubers are used, without the addition of any water.

As stated above, this material can be pulped at a rate of 1 kg. per hour. When the pulp is examined under a microscope it is seen that every cell has been broken except occasional "stone cells" and, in the case of each tuber, the last thin piece of skin which passes between the base of the tube *I* and the wheel; by adjusting the parts carefully the weight of these unground pieces can be rendered negligible compared with the total weight of the tubers used.

When flaccid tubers are to be ground it is necessary to add a little water. Provision has been made for this by means of a narrow brass tube, passing through the plate *D*, close to the spindle and to the tube *I*, through which water or filtered juice may be dropped so as to fall on the rotating wheel and be flung on the material being pulped. This tube is completely plugged up when not actually in use to prevent the formation of a small air-pocket.

The pulp collecting in the bell-jar is filtered by means of alternate layers of filter paper pulp and sand, two layers of the former and three of the latter, supported on a plug of glass wool as shown in the diagram. This gives a filtrate which is only very slightly turbid.

It is found necessary to bubble the carbon dioxide through ether before it enters the bell-jar. In this way the foam into which the wheel "whips" the pulp is discharged.

If the residual pulp be shaken with water and allowed to settle it is possible to separate mechanically:

(1) cell-wall material which floats away readily;

(2) starch grains;

(3) any unground pieces of skin.

These facts suggest that the machine might be used for the separation of cell-wall material for its chemical examination, and that by its means the starch content of potato tubers and similar material might be estimated by a simple mechanical separation.

The apparatus was constructed from the writer's design by Messrs F. and T. Mercer, Apparatus Makers, St Albans, to whom he wishes to express his best thanks for the care and skill exercised by them in its construction.

CXXI. GLUTATHIONE.

ITS INFLUENCE IN THE OXIDATION OF FATS AND PROTEINS.

By FREDERICK GOWLAND HOPKINS.

From the Biochemical Laboratory, University of Cambridge.

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I. Introductory.

This paper gives an account of observations which illustrate certain of the properties of glutathione as a promoter of oxidations. The fresh facts made available are as yet quite insufficient to explain its precise functions in living tissues, but they carry suggestions for future work. The experimental results touch at some points on phenomena concerning which basal chemical knowledge seems lacking. They cannot therefore be succinctly described[1].

Meyerhof has shown that the oxidation of certain unsaturated fatty acids is promoted by the presence of thiol compounds, and the results he obtained by the use of thioglycollic acid and cysteine (in particular the former) are easily reproduced with the dipeptide, though with certain differences that are significant. It will be shown in this paper that a curious limited oxidation of proteins is brought about by the agency of glutathione, exerted in quite peculiar relations. The observations of Miss Thurlow [1925] carried out in this laboratory have shown that in the progress of the oxidation of the SH group peroxide formation occurs, and that under such circumstances, in the presence of a peroxidase, certain secondary oxidations may be induced. On these lines it is probable that the functions of glutathione may come into relation with the events of carbohydrate metabolism.

In the experiments to be described, in so far as they have involved the use of tissue preparations, muscle tissue has been almost exclusively employed. In certain details, though not in fundamentals, the relations of muscle fibres with glutathione are special. The behaviour of cellular tissues requires separate treatment.

Since the publication of the paper by Dixon and myself [1922], which followed the original account of its isolation [Hopkins, 1921], the constitution

[1] Many of the observations described were made more than two years ago, but publication was unavoidably delayed. Unfortunately, more recent work—much limited by lack of leisure—still leaves the subject in a position which is curious and obscure. I think the fresh facts described are of real interest, however, and publication at the present time may stimulate the work of others. The paper should be looked upon, in part at least, as a prolegomenon to publications from this laboratory which will appear in the near future.

of glutathione as diglutaminyl-cysteine has been established on what seems to be conclusive evidence by the work of Quastel, Stewart and Tunnicliffe [1923], and by its synthesis in the hands of the latter two investigators [Stewart and Tunnicliffe, 1925]. The natural product has now been many times prepared (chiefly from yeast) by different individuals, using the method originally described. Szent-Györgyi [1925] has endeavoured to modify that method, but states that he was unable to improve upon it. With experience the original yields have been increased. Some 20 g. of the pure substance from 50 kg. of moist pressed yeast are obtained without difficulty. This falls much below the maximal figure given by direct titration, *circa* 0·2 % [Tunnicliffe, 1925]; but it is certain that serious losses occur during the process of isolation[1]. Tunnicliffe [1925] finds that muscle contains about 0·035 % of which about one-third only has been actually isolated. It is in any case sure that Abderhalden and Wertheimer [1923] are mistaken in supposing that the concentration of free cysteine in the tissues is at all comparable with that of the dipeptide. I possess some evidence to show that the former amounts at the very most to one-twentieth of the latter and is probably much less. Free cysteine cannot play a part of quantitative importance in promoting tissue oxidation.

Any description of the properties of glutathione and any study of its functions must of course take due account of the important observation of Warburg and Sakuma [1923], showing that the SH group of thiol compounds is not strictly autoxidisable, but is oxidised by molecular oxygen only in the presence of minute traces of iron. That this holds in the case of glutathione (or, at least, that the autoxidation of its thiol form is relatively very slow in the absence of all but irremovable traces of iron) has been shown by Harrison [1924]. It would be desirable, therefore, to carry out all experiments with preparations containing a minimal, but exactly known, quantity of the metal. Owing however to the laborious nature of the process required for its complete removal and the waste of highly expensive material involved, either in the removal of the traces present in the preparations used, or in estimating these traces, this course is scarcely possible in practice. Since Warburg's discovery the use of specially pure reagents has yielded preparations containing, at most, infinitesimal quantities of the metal. The employment of these, instead of earlier, preparations has made surprisingly little difference to the experimental results. In any case, this relation with iron does not remove any significance from the presumed functions of the thiol group in respiration. The metal is only another link in the chain. None of the results to which significance is attached in this paper is due to the direct influence of iron alone. This question receives frequent reference; but, otherwise, when the addition of glutathione to a system is mentioned, the presence of such minute amounts of iron as the preparation might contain is presumed.

A statement has been made by Szent-Györgyi [1925] which, if correct,

[1] It is undesirable to attempt to isolate the substance from less than 20 kg. of moist yeast.

could not fail to affect profoundly any views concerning the biological functions of glutathione. This author states that the thiol group as it exists in the tissues (where it is certainly in contact with sufficient iron) is not oxidisable. He prepared an extract from the liver of a guinea-pig by grinding up the organ with specially prepared distilled water. The extract was boiled and filtered. The filtrate gave a strong nitroprusside reaction which did not (apparently) diminish on aeration. He does not mention the p_H of the preparation, and he aerated it for 15 minutes only. But such rapid oxidation is certainly not to be expected under such conditions. It would indeed be disconcerting to find that any factor supposed to be concerned with respiratory oxidations could so rapidly disappear from a tissue preparation of the kind. I have many times seen the nitroprusside reaction completely disappear from unwashed liver tissue suspended in distilled water at p_H 7·4 when shaken in a Barcroft apparatus, but only after 5–6 hours at 37°. For special purposes I have many times proceeded as follows. The liver (or other organ) after grinding (the pulp being first squeezed through coarse muslin) is mixed with twice its volume of distilled water, the mixture made neutral or slightly alkaline, placed in a large stoppered bottle and shaken vigorously in a mechanical shaker. I have several records showing that under these conditions the original intense nitroprusside reaction of an aseptic liver preparation may completely disappear within 8 hours at room temperature. When the preparation is first boiled and filtered the oxidation in the filtrate seems to be a little slower, but with efficient aeration the nitroprusside reaction always finally disappears. Since the publication of the above author Mr Albert Carruthers has repeated this procedure using, instead of ordinary distilled water, water twice distilled in quartz apparatus. This made no difference to the result.

In the discussions which follow, in order to save space and printing, the reduced, or thiol form of glutathione is generally referred to as GSH, and its disulphide form as G_2S_2. It is often convenient to speak of "SH" and "S-S" as though they were independent chemical entities.

II. Experimental Methods.

All the estimations of oxygen uptake were made in the Barcroft "differential manometer" apparatus of the type previously described in publications from this laboratory. The flasks are flat bottomed, shaped so as to provide a large surface for the suspension fluid and secure efficient aeration when the instrument is shaken. It was easy to observe that in none of the experiments described did the rate of oxygen supply become a limiting factor. The instruments were carefully calibrated and four could be shaken side by side by the same motor while immersed in the same bath. Different systems could therefore be compared under identical conditions. Unless otherwise stated, all the measurements were made at 37°. For a reason mentioned in the next section, the phosphate buffer solutions used as suspension fluids in earlier experiments [Hopkins and Dixon, 1922] were given up. As in the great majority of cases

the oxidations studied involved no change in p_H, Ringer solution or distilled water was used. It was found quite easy under these circumstances to adjust the p_H of the systems studied and in the experiments described it was found to be unchanged at the end. The dipeptide (partially or completely neutralised with soda) itself provides slight buffer action.

Many of the results are given in the form of curves relating the oxygen uptake with time. Meyerhof [1923] has correctly remarked that when tissue preparations are shaken in apparatus like the Barcroft manometer, the absolute velocity of uptake may be so greatly affected by accidental factors, such as the physical condition of the preparation, that its measurement has little significance. In certain cases it is doubtless of small value. Nevertheless, when relative, and not absolute, velocities are in question, and in some other cases, curves may give desirable information which is not supplied by a statement of total uptake. When oxidising systems are shaken side by side and when one factor only is varied as between a particular system and a control system, a comparison of the curves obtained is often of value. Those given in the figures are quite unsmoothened.

III. The Oxygen Uptake (with Glutathione) of Washed Muscle and its Thermostable Residue.

In the paper by Hopkins and Dixon [1922] it was shown that when washed muscle is extracted with boiling water, washed with alcohol, dried *in vacuo* and powdered, the dry powder, called in the above paper the thermostable residue, though showing no oxygen uptake by itself, displays a definite uptake in the presence of glutathione. A corresponding amount of the original washed muscle without further treatment is itself capable of the same quantitative uptake, but to establish a sufficiently active system within the swollen and somewhat impermeable fibres of the washed preparation a relatively high concentration of glutathione in the external fluid is necessary. A concentration of the order $M/200$ secures a brisk uptake with the powder. In the case of the washed muscle the uptake with $M/40$ is still relatively slow, but it reaches the same level as that of the powder within a reasonable experimental period (Table I).

Hopkins and Dixon [1922] using Clarke and Lub's phosphate buffer solutions obtained an oxygen uptake of about 400 mm³. per g. of dry powder. It was shortly afterwards found that the high concentrations of phosphate used limit the total oxygen to a remarkable extent, for reasons not yet fully explained. The uptake of an average preparation in Ringer's solution at p_H 7·6 is of the order of 2000 mm³. per g. A great number of such estimations were made at a time when more importance seemed to belong to the actual figures than is really the case. Unselected instances are given in Table I.

Table I.

Oxygen uptake of muscle preparations suspended in aqueous solutions (3 cc.) of glutathione (disulphide). Except where otherwise stated all preparations were in the form of the dry thermostable powder. p_H of system 7·6 in each case.

No. of Exp.	Species of muscle	Weight employed g.	Concentration of G_2S_2	Time of observation hours	O_2 uptake mm.³	Uptake mm.³ per g.	Remarks
1	Cat	0·2	*M*/200	7½	385	1925	
2	Dog (1)	0·2	*M*/200	7½	395	1975	
3	Dog (2)	0·2	*M*/200	8	400	2000	
4	Rabbit	0·2	*M*/200	7	363	1815	
5	Rabbit	1·0	*M*/50	8	285	1425 (on dry weight)	Moist washed muscle—uptake not complete
6	Rat	0·2	*M*/200	7½	454	2270	
7	Frog	0·2	*M*/200	7	310	1550	
8	Frog	0·5	*M*/50	8	110	1100 (on dry weight)	Moist washed muscle—uptake not complete

It was shown in a previous paper that when the dipeptide forms an oxidising system with washed muscle it suffers itself no oxidation save the reversible change in which the sulphur group is alone concerned. The observations in question involved the use of phosphate buffers. It seemed worth while to demonstrate the fact again with the non-buffered media in which the uptake of the system is so much higher. The curves of Fig. 1 show that the total oxygen taken up reaches practically the same value, though of course at different velocities, with greatly varying concentrations of glutathione. These curves were obtained with the muscle powder; but since, as stated above, muscle when simply washed gives with high concentrations of glutathione the same uptake as a corresponding amount of powder with a lower concentration, it is sure that the oxidising agent in washed muscle which oxidises

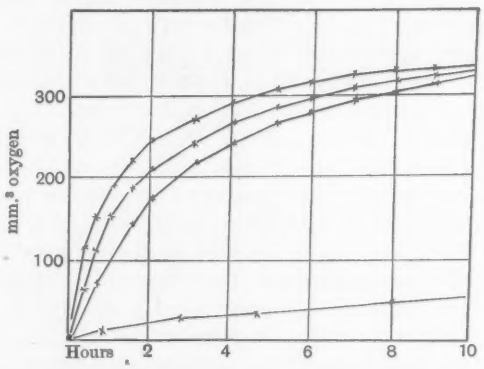

Fig. 1. Oxygen uptake of thermostable powder from cat's muscle, 0·2 g. in 3 cc. Ringer at p_H 7·6. Of the three upper curves the lowest shows the effect of 4 mg., the middle of 8 mg., and the highest of 16 mg. glutathione (G_2S_2). The bottom curve shows the uptake of the powder alone.

free glutaminic acid [Thunberg, 1920] does not affect the glutaminyl moiety of the dipeptide.

Meyerhof [1923], in the important communication which appeared shortly after that of Dixon and myself, also describes observations upon the thermostable residue ("Alkoholpulver"). He reports uptakes (with thioglycollic acid) as high as 5000 mm.^{3} per g., though many of his data point to a lower figure than this. A comparison of his figures with my own is of small significance, however. Many of them were obtained under different conditions, especially in respect of the hydrogen ion concentration in the systems studied. Variations in this affect more than velocity of uptake. It will be shown, for instance, that in acid systems the oxidation of fat is favoured, whilst oxidation of quite other material proceeds best at the neutral point or in slightly alkaline solutions. A difference in hydrogen ion concentration will therefore affect the result both quantitatively and qualitatively (see Figs. 9, 10 and 11, p. 813, section VI). Meyerhof used thioglycollic acid. In the experiments of Table I glutathione was employed not as the thiol compound, but in its disulphide form.

As a matter of fact, the significance of the oxygen uptake of the thermostable residue is somewhat different from what may have been suggested or implied in previous publications. It will be convenient, before returning to it, first to consider the behaviour of systems containing unsaturated fats and glutathione alone, and afterwards that of fat-free tissue-systems. The former take up oxygen only when the thiol form of the dipeptide is present from the first, since the fats do not reduce the disulphide form. Oxidation in the latter proceeds with either form.

IV. The Oxidation of Unsaturated Fatty Acids and Fats.

In aqueous systems containing GSH together with an emulsion of unsaturated fatty acids (linolenic, linoleic), so long as the p_H is of the order of 3·0 and 4·0, the concentration of the SH group is long (though not indefinitely) maintained during the aeration of the system. Oxygen many times in excess of what is required to oxidise the SH to S-S is taken up and a corresponding amount of fatty acid oxidised. When, however, the hydrogen ion concentration approaches the neutral point, or when it is near to this on the alkaline side (p_H 7·4–7·6), the behaviour of such systems is profoundly changed. The SH group (the presence of traces of iron being presupposed) is then so freely oxidisable that, unless its concentration in the system be relatively high, it rapidly disappears, even in the presence of the fatty acid. The system then becomes inert before more than relatively small amounts of fatty acid have been oxidised. The behaviour of systems composed of GSH and unsaturated fatty acids seems to be therefore essentially the same as that described by Meyerhof for the systems lecithin-thioglycollic acid and linolenic acid-thioglycollic acid. (See Table II and compare the results of Exps. 1–5 with those of Meyerhof [1923, Table XV, p. 548].)

In considering acid systems in which the concentration of the SH group

falls off very slowly during the aeration of the system, the view of Meyerhof [1923, p. 561] as to the mechanism of the oxidation seems adequate. It will be remembered that he postulates the preliminary formation of a peroxide by two thiol groups. This peroxide is assumed to decompose then in such a way that the oxygen is wholly transferred to the unsaturated linkages of the fatty acid, whilst the original SH groups are reconstituted. The oxidation of the former is what would usually be considered as a catalysed reaction and the thiol group is concerned essentially with oxygen transport.

Table II.

Ref. no.	Material employed 10 mg. as emulsion in distilled water	p_H	Time for uptake hours	Total uptake mm.³	O_2 equivalent of SH present mm.³	O_2 to fat mm.³	Remarks
1	Linolenic acid	3·5	6	210	32	178	Acid systems
2	,,	3·8	6	225	41	184	In all cases the uptake was still progressing at end of observation
3	Linoleic acid	3·5	6	140	32	108	
4	Mixed acids from linseed oil	3·2	5½	710	200	510	
5	Mixed acids from linseed oil	3·5	8½	550	90	460	
6	Linolenic acid	7·6	6½	40	26	14	Neutral or alkaline systems
7	Mixed acids	7·4	7	62	35	27	
8	,,	7·6	7	830	425	405	
9	,,	7·6	4	180	90	90	
10	,,	7·6	3	325	165	160	Uptake complete in all cases
11	Linoleic acid	7·4	5	250	132	118	
12	Lecithin	7·6	6½	732	390	342	

It is important, however, to consider in greater detail the behaviour of the system GSH + unsaturated fatty acid at p_H 7·4–7·6. Meyerhof states that at the neutral point the oxidation of fat under the influence of thioglycollic acid is completely inhibited. With reduced glutathione this is not the case. I have found as the result of a number of observations that within a wide range of SH concentrations the oxygen uptake of the fatty acid, or its sodium salt, is equal, or approximately equal, to the amount of oxygen required to oxidise the SH. In other words, when the reaction ceases, the total uptake proves to be twice the oxygen equivalent of the SH present. To show this by the use of the Barcroft apparatus, it is necessary to work with relatively large concentrations of GSH. If small amounts are used, such as are ample for the demonstration of the more strictly catalytic effect at p_H 3·5, the total uptake at 7·6 becomes too small for accurate measurement, though the relation in question may nevertheless be indicated (Table II, Exps. 6 and 7). Moreover, unless the equilibration of the instrument at the beginning of the experiment is carried out with the flasks filled with nitrogen (oxygen being admitted only after equilibration), some oxidation of the SH group may occur before the system is, so to speak, properly established. This error does not count for much when the total amount of glutathione is sufficiently large. The figures of Exps. 6–12 of Table II show that the relation mentioned above holds within the limits of experimental error at p_H 7·4–7·6. It is the familiar relation found so often when the autoxidation of one molecule induces the oxidation of a

second non-autoxidisable molecule. The SH group is, in neutral or slightly alkaline systems, no longer an oxygen carrier in the sense that Meyerhof postulates for acid systems. It would seem as though its case becomes one represented by the first of the alternative schemata which have been so familiar from the publications of Engler and his colleagues; *A* being an Autoxidator, and *B* an Acceptor.

$$(1)\ AO_2 + B \longrightarrow AO + BO; \qquad (2)\ AO_2 + 2B \longrightarrow A + 2BO.$$

The first is an induced or coupled oxidation; the second is a catalysed oxidation. Whether it be justifiable or not to claim that the change in hydrogen ion concentration actually changes the type of reaction from (2) to (1), there is no doubt that in neutral or slightly alkaline solutions the oxidation of the SH group induces a limited oxidation of the unsaturated fatty acids, and, at the same time the figures obtained strongly suggest that equipartition of oxygen actually occurs. Such cases are at any rate common. The simple schema (1) above may not, of course, properly represent the details of the reaction. Without knowledge, however, as to what happens at the unsaturated linkages of the fatty acids as the result of the limited oxidation in question (it is not necessarily the same as what occurs in more acid systems), it is impossible to say whether or not there are chemical grounds against the above assumption.

It is at any rate of some interest to know that the oxidation of fats is influenced by glutathione at hydrogen ion concentrations near to that of the tissues as well as at others more remote.

The figures of Table II and the curves of Figs. 2 and 3 show plainly the effect of change of p_H on systems comprising GSH and fatty acids.

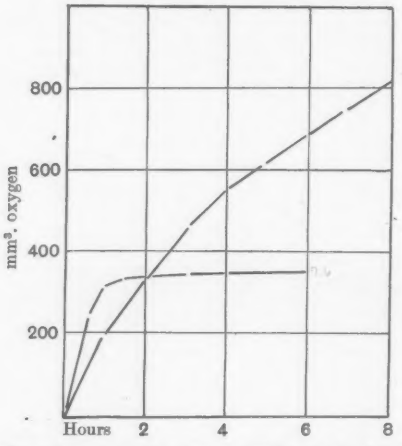

Fig. 2. Showing effect of p_H upon the oxygen uptake of mixed fatty acids from linseed oil in presence of GSH. Prolonged uptake at 3·8; short uptake at 7·6. Acids 10 mg. in emulsion. Oxygen equivalent of SH present 182 mm³. The fatty acids by themselves showed no uptake

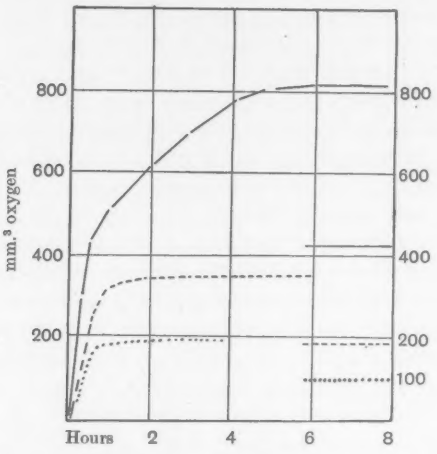

Fig. 3. Oxygen uptake of fatty acids with varying amounts of GSH at p_H 7·6. The horizontal lines mark the oxygen equivalent of the SH present in each case.

It is perhaps desirable to refer here to a point in the technique used for these experiments which might seem to make it inadequate. When, in any combination, fat emulsions are shaken with air, the degree of dispersion of the fat phase might be expected to prove a factor in the velocity of oxygen uptake so dominant as to obscure the effect of other variants. This is not the case. In practice it has proved easy to reproduce any given experimental result, including even a velocity curve, without any special effort to standardise the emulsion, save in respect of the amount of fat present in it and the p_H of the system. Once the preparation is measured into the flask of the Barcroft apparatus, the shaking of the instrument maintains dispersion of the fat, and when other conditions have been kept constant, it has proved easy to obtain accurate reproductions of a given result on successive occasions, without giving more than ordinary care to the rate of shaking. When in the experiments described emulsions at p_H 7·4–7·6 were under study, small quantities of sodium carbonate were used to adjust the reaction and sodium salts were therefore present from the first. In this case also smooth and reproducible curves of uptake were obtained. It is remarkable that in such systems the surface relations should appear to play a relatively minor part in determining the velocity of oxidation.

It is clear that the significance of the results so far described is in any case quite independent of the absolute velocity of oxygen uptake. The curves of Figs. 2 and 3 satisfactorily illustrate however the complete change of type which the oxidising system undergoes on change of p_H.

In observations dealing with the oxidation of linseed oil emulsions, to which reference will be made immediately, changes of velocity during the course of uptake are assumed to be of chemical significance. I could find indeed no evidence whatever to suggest that changes in the grade of dispersion or other non-significant factors played any part in them.

In parenthesis, it may be here remarked that although minute traces of iron (such as were doubtless present in the preparations used) may be necessary for the autoxidation of the SH group of glutathione, none of the results described is brought about by the direct effects of the iron as such. If (as I have repeatedly found) any previously active preparation of the reduced dipeptide

be oxidised by aeration to the disulphide form, it then induces no observable oxygen uptake by the fatty acids, although of course its iron content is intact. An ionised salt of iron will doubtless in weakly acid systems catalyse the oxidation of linolenic acid at concentrations which, in an absolute sense, are low; but to produce an observable effect the concentration must be much larger than that introduced with the glutathione into the systems studied. At p_H 7·4–7·6 iron by itself fails to promote oxidation of the fatty acids, or their alkali salts, and the point does not arise.

It is a remarkable fact that the course of oxygen uptake by the glycerides of linoleic and linolenic acids, under the influence of GSH, differs in several particulars from that exhibited by the free fatty acids or their sodium salts. This statement is based upon experiments done with the purest obtainable specimens of linseed oil.

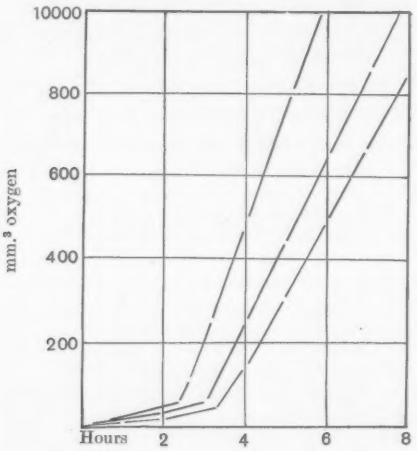

Fig. 4. Uptake of linseed oil (20 mg. in emulsion) with GSH at p_H 3·5. Left-hand curve with 12 g., middle curve with 8 mg. GSH. Right-hand curve 8 mg., but with oil first boiled *in vacuo*.

In acid systems (p_H 3·5–4·0) the curve of uptake of the system (GSH + linseed oil emulsion) shows a marked "period of induction," during which the system is almost completely inert. This period generally lasts from 1–3 hours, being diminished, but never abolished, by increasing the concentration of GSH. It is diminished by previous aeration of the oil, and lengthened if the oil be first boiled *in vacuo* (see curves of Fig. 4). These phenomena are of course familiar from studies concerned with inorganic "driers," or "siccatives," used technically to accelerate the drying of linseed oil in paints and varnishes. The fact that the progress of oxygen uptake during the drying of the oil yields the sinuous time curve (with its period of induction) characteristic of an autocatalytic reaction has been established by many observers. Evidence suggests

that the autocatalyst involved is an autogenous peroxide which when the fat is aerated alone only very slowly reaches an effective concentration. Rideal [1919], Coffey [1921], and others have shown that the period of induction persists, though much shortened, in the presence of driers. Agents which promote the oxidation of linseed oil may therefore be "pseudo-catalysts" serving to assist in the formation of (or in maintaining the concentration of) the autocatalytic peroxide. The curves of Fig. 4 obtained at p_H 3·5 suggest that at least when glycerides are concerned the thiol compounds come into the same category[1]. I have never succeeded in obtaining evidence of an induction period in the case of the free fatty acids, however treated, or at any p_H. If Meyerhof's view, as stated above, concerning the oxidation of the free acids by SH compounds be correct, and in acid systems it seems fully justified, it would seem that the mechanism of oxidation is actually different in the case of the fatty acids, or their sodium salts, and that of their glycerides, respectively. I cannot find from the chemical literature that any study of the effect of ordinary driers upon the free acids has been made[2].

At p_H 7·4–7·6 linseed oil again shows a behaviour which is strikingly different from that of free linoleic and linolenic acids.

When an emulsion of the oil is shaken in the presence of relatively high concentrations (M/100–M/50) of GSH, in a neutral or weakly alkaline medium, the equipartition of oxygen so characteristic of systems containing the free fatty acids is not seen. The curve of oxygen uptake assumes a characteristic form, which has shown itself constant in a great number of observations (Fig. 5). There is no period of induction. The high initial velocity falls off somewhat rapidly and gives place to an uptake with a lower velocity which remains exactly linear for several hours and until the fat is nearly all oxidised. The inflexion in the curve occurs when sufficient oxygen has been absorbed to correspond nearly (but not quite) with that required to oxidise the SH present. During the linear period the concentration of SH is therefore very low, a circumstance which has been repeatedly confirmed by the use of the nitroprusside reaction. It would seem that during the first stage of uptake the SH group of the glutathione is oxidised without any associated effect upon the fat. The impression is nevertheless given (though it is not necessarily correct) that an efficient concentration of the thiol group must survive long enough to secure the establishment of some condition in the system which is not found initially. When quite small concentrations (less, say, than M/200)

[1] If so their influence upon the unsaturated linkages must be somewhat different from that exerted when they are oxidising fatty acids. In spite of the general assumption it is not proven that the autocatalyst is necessarily a peroxide. The following observation has some interest. One of the samples of linseed oil used in my experiments was aerated alone in the form of an emulsion for long periods. After 12 hours it showed no tendency to autoxidise. After 20 hours however when a sample of the emulsion was transferred to a Barcroft apparatus it displayed straightway without any addition a slow but maintained uptake of oxygen. This was at once greatly accelerated on the addition of a little GSH.

[2] Armstrong and Hilditch [1925] have shown that the simple esters (of oleic acid) are much more resistant to the action of oxidising agents than is the free acid.

are present initially, the oxidising system may not be established. The SH is rapidly oxidised and all uptake may then cease.

The longer the induction period displayed by a sample of oil at p_H 3 to 4, the higher the concentration of SH required to establish an active system at p_H 7·6.

What then is the actual state of affairs during the highly characteristic linear uptake? Is the oil at this stage undergoing autoxidation, the period of induction having been much shortened by the conditions, and obscured by the preliminary oxygen uptake of the SH group? Or, is the efficient promoter of oxidation during the linear period a highly active compound formed by the traces of iron present with that very small residual amount of glutathione which is found to exist in the system during this later stage of the oxygen uptake?

The direct promotion of oxidation by iron cannot be ignored here, because, unlike that of fatty acids, the oxidation of linseed oil is definitely promoted by moderate concentrations of iron in neutral or slightly alkaline systems. It is sure, however, that the minute amounts present in the glutathione preparations used are not by themselves responsible for the phenomena under discussion, since when preparations of the dipeptide are oxidised by aeration they have no effect upon the system when added during any stage of the course of uptake, though the iron content is not altered.

In oil emulsion systems the use of cyanide to inhibit any possible influence of iron offers certain difficulties. The perfectly smooth curves obtained in its absence are apt to become irregular. It is certain, however, that cyanide does not prevent the oxidation of linseed oil under the influence of glutathione preparations at p_H 7·6. When special care has been taken to avoid any change in the p_H as the result of its addition, I have found that even with concentrations of cyanide so high as $M/12$ the effect has been relatively slight. Curves have been obtained precisely similar in form to those seen without cyanide, though usually the velocity of the linear period is somewhat lowered (Fig. 5).

I have obtained no evidence upon which to base any clear explanation of the behaviour of these glyceride systems when neutral or slightly alkaline; but the facts which indicate such marked differences in the behaviour of free fatty acids, or their sodium salts, and their glycerides, respectively, when undergoing oxidation (as emulsions) under the influence of an agent present in living cells, seemed to deserve record. It is of some biological interest to know that lecithin behaves like the former rather than like the latter.

(The data were obtained by the use of three different samples of linseed oil, a sample of pure linolenic acid from Kahlbaum—of which I possessed only a small quantity and could obtain no more—a supply of pure linoleic acid (Kahlbaum) and one of mixed acids, of iodine value 162, carefully prepared from linseed oil[1] and given me by Mr N. K. Adam.)

[1] It is clear that the observations upon linseed oil described in this section should be repeated upon purified individual glycerides.

V. Oxygen Uptake (with Glutathione) of Lipoid-free Preparations.

As the result of his studies with thioglycollic acid and cysteine, Meyerhof held that the induced oxygen uptake of a thermostable preparation of muscle after thorough extraction with alcohol and ether is so small as to lack significance. I have no doubt that this was mainly due to the hydrogen ion concentration of the systems he studied[1].

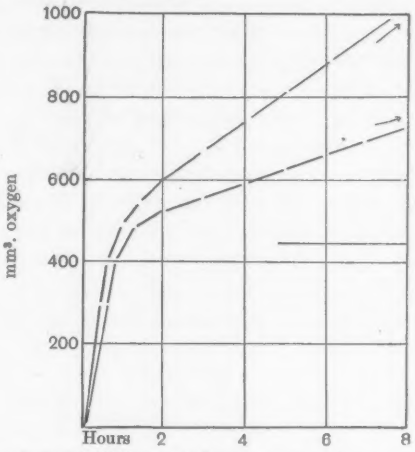

Fig. 5. Uptake of linseed oil with GSH at p_H 7·6 (upper curve). Lower curve the same in presence of $M/12$ KCN. The horizontal line indicates the oxygen equivalent of the SH present. The uptake remained strictly linear till the 15th hour when the observation ceased.

A preparation from washed muscle after very thorough extraction gives in the presence of glutathione at p_H 3·5 no uptake at all. At p_H 7·6, however, it displays an uptake which is never less than one-third of that of an unextracted preparation. It may, indeed, constitute a considerably larger fraction of the latter.

This noteworthy effect of change in p_H shows that the material oxidised in the extracted material is not residual lipoids. It bears also on the nature of the mechanism involved.

Two precautions in technique increase the uptake of such preparations. The first relates to the method of washing the original muscle. If, as recommended by Thunberg in his classical paper on hydrogen donators [1920], the chopped muscle be shaken with water in stoppered cylinders, it is essential that the first washing should be with as large a bulk of fluid as possible and that this should be quickly renewed. So long as the concentration of glutathione in contact with the muscle is appreciable (and most of the substance comes out in the first washings) shaking with air involves an oxidation[2] which appreciably reduces the uptake of a final preparation. Later washings than the first have

[1] Meyerhof [1923, p. 542] does not mention the p_H of his media in this section of his paper, but it is likely from internal evidence that the observations were made on the acid side of neutrality.

[2] The "fixed SH" group is partly oxidised.

much less effect. The second point in technique is to avoid oxidation due to peroxide formation in the ether in the residue undergoing extraction. In my own experiments thermostable muscle powders were extracted in a Soxhlet apparatus, first for 8 hours with absolute alcohol; then for a similar period with thoroughly dry ether, and finally for a few hours with alcohol again. The ether is thus removed before the preparation is taken out of the Soxhlet tube. The alcohol is removed in a vacuum desiccator.

In Fig. 6 will be found typical illustrations of the oxygen uptake at p_H 7·6 of lipoid-free preparations obtained as above. None of them showed any appreciable uptake in the absence of glutathione. It should be observed that all the curves of Fig. 6 were obtained by adding the glutathione in its disulphide form. This means, as will be later more fully demonstrated, that the extracted, lipoid-free tissue retains intact the ability to reduce the disulphide, a circumstance which together with the special influence of the hydrogen ion concentration already mentioned becomes of importance when the nature and mechanism of oxidation in the muscle residue are being considered.

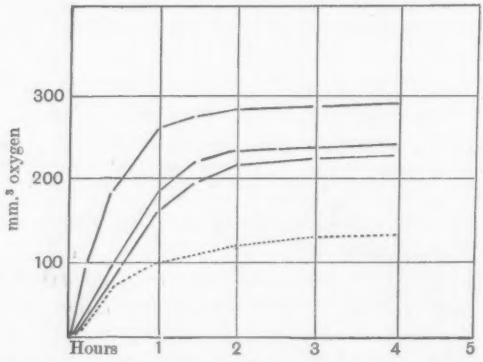

Fig. 6. Uptake of lipoid-free muscle preparations (0·2 g.) at p_H 7·6 with G_2S_2 (M/200). The dotted curve was obtained from the preparation which originally yielded the middle curve. It had been kept at room temperature for 3 weeks.

It will be later shown that the oxidation involves the proteins of the tissue. As however most of the observations were made upon muscle before this fact was known, it will be convenient to ignore it for a moment, while some experimental results are discussed in terms of the muscle residue as such.

The oxidation of the fat-free material which is induced by contact with glutathione depends in a remarkable way upon the existence in the former of what was described by Dixon and myself [1922] as the "fixed SH group." It is especially characteristic of muscle as a tissue that after the whole of the soluble glutathione has been removed from it by thorough washing, the insoluble residue still gives a strong nitroprusside reaction. It may perhaps be said that no absolute proof has been given that this reaction is, as a matter of fact, due

to an SH group. By a process of exclusion, however, and more particularly by consideration of certain facts dealt with in this paper, the assumption will be found to be justified. According to Abderhalden and Wertheimer [1923] the intensity of the reaction is increased in rigor mortis and diminished by denervation of the muscle during life.

It is essential to any understanding of the oxidations controlled by glutathione, that certain facts concerning the fixed SH group[1] should be fully described. They are curious and necessitate a somewhat detailed discussion.

The fixed SH group is resistant to oxidation by molecular oxygen. Washed muscle or a powdered thermostable residue giving a strong nitroprusside reaction may be suspended in water, or a buffer solution, at p_H 7·6 and aerated for many hours without any appreciable change in the intensity of the reaction. When aerated, however, under conditions otherwise similar but in the presence of glutathione (GSH, or G_2S_2; 50–100 mg. per g. of tissue) the colour reaction progressively diminishes, till after 5 or 6 hours at 37° no reaction is given either by the tissue or the fluid in which it is suspended.

Moreover, the tissue, even when under strictly anaerobic conditions, can with equal ease be deprived of its power to yield the nitroprusside reaction. If it be suspended in an aqueous solution of oxidised glutathione at p_H 7·6 in a fully evacuated vessel the reaction is found progressively to disappear from the tissue, while, under these anaerobic conditions, the solution, which of course gives none at the outset, comes to display an intense reaction.

It is important, however, to make it clear that in this phenomenon equilibrium relations are displayed. The transference of the colour reaction (to use for the moment a non-committal phrase) from tissue to solution may cease while it is incomplete. Before the tissue ceases to give any observable reaction, it must have been exposed to G_2S_2 not only for a sufficient time, but to a solution of sufficient volume and concentration.

> The following describes an observation, typical of many, illustrating the above-mentioned facts. 1 g. of thermostable muscle powder giving a strong nitroprusside reaction was placed in a vacuum tube with 20 cc. of $M/100$ G_2S_2. The tube was evacuated and allowed to stand, with occasional shaking, for 3 hours at 37°. On opening the tube the fluid was found to give an intense reaction, whilst a sample of the tissue after thorough washing yielded one which was still definite, though relatively slight. The tissue and fluid were then returned to the vacuum tube which was again exhausted. No further change occurred; the system was already in equilibrium. The original fluid was then replaced by a second quantity (10 cc.) of G_2S_2 solution. After 2 hours the solution gave a slight reaction, whilst the washed tissue now gave no reaction strong enough for observation.

A tissue preparation which, as the result of the treatment described, gives no observable nitroprusside reaction again yields an intense reaction after contact with solutions of thiol compounds in sufficient concentration. This restored colour reaction, like the original, is still displayed with undiminished intensity after the most thorough washing of the tissue with water.

[1] The term is not a particularly happy one, but it is convenient in discussion. It has proved necessary to use it very frequently in this paper.

Effective in producing the above result are solutions (*circa* $M/10$) of reduced glutathione, cysteine, and thioglycollic acid. As will be later shown, the intensity of the reaction as thus restored increases up to a limit with the concentration of the SH solution to which the tissue has been exposed. There is no doubt that a "fixed" SH group is in this way restored to any tissue preparation which by contact with the disulphide form of glutathione has previously lost this factor. Like that in the original tissue, the restored group is, as already stated, quite irremovable by washing. It is resistant, like the former, to direct oxidation, but disappears in like manner as the result of contact with solutions of G_2S_2. By repeating the treatment just described it may be again restored. A fact of special note is that a muscle preparation which on the loss of its original fixed thiol group ceases to take up oxygen in the presence of glutathione is, after the restoration, capable of a renewed uptake greatly in excess of the oxygen equivalent of the SH restored. This matter is fully dealt with later.

An endeavour has been made to throw further light on the nature of the phenomena just described. On reading the above description, or on observing the phenomena for the first time the impression might be left that the thiol compound used to restore the SH group is merely adsorbed by the tissue residue; or—since the "restored" group in the latter withstands all washing—that it has become fixed at some special surface in the muscle. If this view were justified, it might be further assumed that the original nitroprusside reaction of washed muscle is due to "fixed" glutathione. The distinction between the soluble dipeptide, which can be so readily washed out, and the molecule, or structure, carrying the SH group, which no amount of washing affects, is doubtless sharp. It must be remembered, however, that the muscle fibre is heterogeneous. While the greater part of the glutathione may be simply dissolved in the fibres, a part might be fixed owing to the properties of a special surface. When by contact with solutions of G_2S_2 the nitroprusside reaction disappears from the tissue and reappears in the solution, it might be because the disulphide form, when in sufficiently high concentration, is able to replace the thiol form (or another thiol compound) at the surface in question. Such a view is perhaps unlikely, but I find as a result of discussing the facts with others that it is necessary to consider it. It can easily be disproved, however.

A tissue preparation which has lost its nitroprusside reaction as the result of contact with a solution of G_2S_2 will again show a reaction upon treatment with sulphites. The best result is got by warming it with sodium hydrosulphite. The reaction is never so intense as when it is restored by exposure to solutions of GSH, etc., but it is similarly removed by fresh contact with a G_2S_2 solution which then itself shows a typical reaction. Hydrosulphites induce colour changes in nitroprusside solutions, but give no reaction which could possibly be confused with that of the SH group. When a nitroprusside reaction has been restored to the tissue by exposure to a thiol compound and when the

reaction is found to be transferable to a solution of G_2S_2, it is easy to show that this is not because the latter liberates the thiol compound from some previously established association with the tissue. Clear proof can be obtained by the use of thioglycollic acid. This substance is extracted from acid aqueous solution by ether, but can be easily removed from the ether by shaking with a small volume of alkaline water. In the circumstances of the experiments under description, it is therefore easy to decide whether it be or be not present in a G_2S_2 solution. Neither form of glutathione is soluble in ether.

The nitroprusside reaction was restored to a muscle preparation (2·0 g.) by exposure to a thioglycollic acid solution ($M/10$) at p_H 6·8 for 3 hours. After the usual thorough washing, it was suspended anaerobically in 10 cc. of G_2S_2 solution ($M/50$, p_H 7·4) for 3 hours at 37°. The solution, which then showed an intense reaction, was made slightly acid with sulphuric acid and shaken with 10 times its volume of ether. The ether after filtration through three dry filter papers, to remove traces of the aqueous phase, was then shaken with 10 cc. of water made slightly alkaline. The extract, after filtration through two wet filter papers to remove traces of the ether phase, was tested for the nitroprusside reaction. In spite of its extreme delicacy the reaction was entirely absent. At least 8 mg. of thioglycollic acid must have been present, were this substance responsible for the amount of SH in the solution; whereas in a control experiment, when 3 mg. only were added to the same bulk of G_2S_2 solution and the above processes applied, the aqueous extract from the ether gave an intense nitroprusside reaction. This experiment has been many times repeated; always with the same result[1].

It is clear that in the experiments just described the observed transference of the nitroprusside reaction from tissue to solution could not have been due to the replacement of adsorbed thioglycollic acid. Now the general properties of the fixed SH group are the same in the original washed muscle, and in preparations to which it has been restored by contact with solutions of thioglycollic acid, cysteine, or reduced glutathione.

It is sure, therefore, that the phenomena under discussion are not due to interchange between molecules in solution and molecules fixed under reversible conditions in the tissue. The only alternative is to recognise that they involve actual oxidation and reduction. The carrier of the fixed SH group in the tissue must be able to reduce glutathione when, as a disulphide, it is present in solution, and itself undergo oxidation by the latter. On the other hand, the tissue factor when oxidised (presumably to a disulphide form) can upon change of concentrations be reduced by thiol compounds in solution. It must, of course, be remembered that the molecular associations of the reacting sulphur groups are not the same. The result of such reversible relations must be the establishment of equilibria depending upon the relative concentrations of SH and S-S in solution and the activity of similar groups in the solid phase.

With reversible reactions occurring in a heterogeneous system of this kind equilibrium conditions must be complex, and their quantitative study (especially having regard to the nature of the materials) by no means easy. Some estimations dealing with the exchange of the SH group between the solution and the tissue have been made, however.

[1] I owe to Mr D. C. Harrison the suggestion that the properties of thioglycollic acid would make the above test available. When it is used as above to reduce a tissue preparation, prolonged washing is required for the removal of adsorbed traces.

Oxidised tissue preparations giving no nitroprusside reaction were exposed to solutions of cysteine, reduced glutathione or thioglycollic acid in varying concentrations. By means of titrations with iodine ($N/100$) the diminution in the SH group of the solutions was determined. Since the concentrations were sometimes high and the change relatively small, estimations by difference were not satisfactory. Moreover, when the observations are made at p_H 7·6, the thiol compounds are apt to oxidise during the processes of measurement, etc. The tissue preparations were therefore first reduced by anaerobic exposure to a solution of the thiol compound and after thorough washing were suspended, also under anaerobic conditions, in a solution of the disulphide form of glutathione, sufficient in amount to secure complete oxidation of the SH group in the tissue. As the amount of SH which then appears in the solution is proportionate to, and, as will later be pointed out, probably identical with that previously in the tissue, direct titration of the solution gives the information required. The following are typical observations.

A thermostable muscle powder in quantity was oxidised until it gave no trace of a nitroprusside reaction, and was then washed, dehydrated with alcohol, and re-dried in a desiccator. 1 g. was placed in each of a series of vacuum tubes containing 5 cc. of aqueous solutions of thioglycollic acid of varying concentrations (see below). The acid was neutralised with sodium carbonate (p_H 7·6). After 3 hours in evacuated tubes at 37°, the tissue residues were filtered off and very thoroughly washed, until the washing gave no trace of a nitroprusside reaction. Each lot was then placed in a fresh vacuum vessel and covered with 20 cc. of G_2S_2 solution ($M/50$). The tubes were evacuated and stood with occasional shaking for 3 hours at 37°. They were then opened and their contents immediately made slightly acid with sulphuric acid to stabilise the SH group. The tissue residues were then filtered off and washed, and the filtrates titrated with $N/100$ iodine; starch being used as an indicator. The results were as follows:

Concentration of the thioglycollic acid solutions	$M/100$	$M/50$	$M/20$	$M/15$	$M/10$
cc. of $N/100$ I_2 required	0·95	1·90	3·3	3·7	4·7

Although the titration figures in this experiment are small, they are reliable and consistent with the results of other experiments of the same type. They lie on a smooth curve and when cysteine or glutathione was used to reduce the oxidised tissue similar curves were obtained.

In one experiment G_2S_2 solutions of different concentrations were placed in contact with equal weights of muscle powder (containing its original fixed SH) and, after equilibrium was established anaerobically, the GSH in solution was determined, as before, by iodine titration.

	$M/100$	$M/50$	$M/50$	$M/10$
Volume and concentration of G_2S_2 in contact with 1 g. muscle residue	5 cc.	5 cc.	10 cc.	5 cc.
cc. of $N/100$ I_2 required	2·4	3·1	3·4	4·2

The figures of these experiments are reproduced as they demonstrate clearly the existence of equilibria between glutathione in solution and sulphur groups in the insoluble muscle residue. No more than this is claimed for them.

The important influence of the fixed SH group upon the oxygen uptake of the lipoid-free muscle residue now calls for discussion. There is no uptake

without contact with glutathione in solution; there is also no uptake without the existence of the SH group in the tissue itself. Seeing then that the disappearance of the latter is so characteristic an indicator of oxidation by G_2S_2, the suggestion might arise that its oxidation is responsible for the whole oxygen uptake. This however as will be shown immediately is certainly not the case.

On the other hand, it seems likely that the SH group is the only reducing agency left in the thermostable residue, and probably the only factor, even in muscle which has been simply washed, which reduces glutathione. Tunnicliffe [1925] found that the oxygen equivalent of the SH which appears in G_2S_2 solutions after reduction by 1 g. of thermostable muscle powder is of the order of 140–150 mm.^{3}. Such figures I have frequently obtained. When, however, the original muscle has been washed with the precautions mentioned at the beginning of this section, and when (remembering the equilibrium relations) care is taken to see, by obtaining a negative nitroprusside test, that the tissue is completely oxidised[1], the above value comes nearer to 200 mm.^{3}. I do not find that it is altered by removal of the lipoids if care be taken to avoid oxidation during extraction by ether. Now, if one estimates by iodine titration, with such accuracy as is possible, the oxygen equivalent of the SH which disappears from a strong GSH solution (M/10, or higher) when employed to restore the SH group to 1 g. of an oxidised preparation, it again proves to be of the same order. That the SH group alone is the essential reducing factor in the thermostable muscle residue, and that the above data give therefore a measure of its influence become nearly certain when the phenomena under discussion are found as will be immediately shown to depend upon the properties of protein.

If the thiol group be the sole reducing factor acting in the system constituted by the muscle residue and glutathione, it is certain that the capacity for oxygen uptake of the system is greatly in excess of the 200 mm.^{3} per g. dry tissue which represents the oxygen equivalent of that group. The former, as we have seen, is of the order of 800–1000 mm.^{3} per g. or even more. Moreover, it is limited to this amount solely because during the oxygenation of the system under the experimental conditions the fixed SH becomes itself oxidised long before other processes of oxidation which it promotes, and for which its presence is necessary, are complete. If a muscle residue, which has been oxidised by G_2S_2 on lines which have been fully discussed, so that it no longer gives a nitroprusside reaction, and, in consequence, has ceased to take up oxygen, be again completely reduced by contact with a strong solution of GSH, etc., and thoroughly washed, it is found to display a fresh uptake which may be of the order of 2000 mm.^{3} (in the case of lipoid-free preparations) per gram. The amount of this uptake depends upon the concentration of the thiol solution to which the preparation has been exposed; that is, upon the

[1] Szent-Györgyi [1925] assumes that the fixed SH is not oxidised by G_2S_2. He evidently did not observe that the reaction comes to an equilibrium and in any case takes time to complete.

extent to which it has been reduced and its fixed SH group restored. The above figure is obtained after contact with GSH in solutions of $M/10$, or upwards. After this second uptake has ceased, fresh reduction again induces a fresh capacity for the absorption of oxygen (always necessitating the presence of glutathione) which may again amount to a figure of the above order! It will now be understood that the actual uptake of a lipoid-free muscle residue as first prepared will depend upon whether its fixed SH group is at a maximum or not.

The following table illustrates the nature of the quantitative data which have been obtained during a study of the phenomena in question. Preparation A was a fat-free thermostable powder, prepared without special precautions and used some weeks after its preparation. In the preparation of B, an otherwise similar powder, care was taken at each step to avoid oxidation, and it was used immediately.

Table III.

Thermostable preparations from rabbit's muscle, extracted with alcohol followed by ether. "A" a stock preparation, "B" a preparation made with careful avoidance of oxidation (see text) and used when fresh.

Treatment of material before determination of uptake	Total O_2 uptake when shaken in G_2S_2 solution ($M/200$) at p_H 7·6; mm.³ per g.	
	A	B
Original preparation before removal of lipoids	2100	—
Extracted (alcohol-ether-alcohol)	710	1020
Oxidised acrobically with G_2S_2 till free from nitroprusside reaction	±40	0
Reduced in GSH solution ($M/10$)	1010+	1850
Oxidised anaerobically with G_2S_2	0	—
Reduced in cysteine solution ($M/10$))	1810	—
Oxidised aerobically and then reduced by thioglycollic acid ($M/10$)	—	2010

V *a*. Uptake of pure Proteins.

It will now be shown that the special characters of the fat-free muscle residue which have just been discussed are due to the properties of its proteins, and that the oxidations which occur in it involve apparently no other materials.

Earlier observations bear upon the matter, but I think the significance of the known facts is much increased in the light of the observations to be described.

Arnold [1914] observed that muscle proteins give a nitroprusside reaction, though at the time it was somewhat of an assumption that this was due to the proteins as such. Especially intense, he found, was the reaction in the case of the least soluble proteins (the "myostromin" of Danilewski). The proteins of blood plasma and those of "excretions and secretions" gave no reaction. Heffter [1907] had previously shown that blood proteins, serum globulin at

any rate, can be made to yield a reaction on treatment with sodium sulphite. Gelatin, caseinogen, egg-globulin and fibrin did not display this property.

Heffter showed that egg-albumin, which gives no reaction in its natural state, acquires the power to do so after denaturation. This remarkable accompaniment of denaturation formed the subject of an extensive study by L. J. Harris [1923] in this laboratory. He advanced some suggestive hypotheses in explanation.

Abderhalden and Wertheimer [1923] have made the interesting observation that when egg-albumin is coagulated by heat, and shows in consequence a nitroprusside reaction, it can form (with cystine) a system which takes up an appreciable amount of oxygen (45 mm.^{3} per g. per hour). The total uptake, however, is, I find, very small.

The nitroprusside reaction as conferred upon certain pure proteins when, as coagula, they are brought into contact with strong solutions of thiol compounds is very much more intense than can be produced by denaturation processes alone, even in the case of ovalbumin, which among native proteins offers the only case of a marked reaction developing on simple coagulation by heat. The proteins of blood serum which develop no trace of a reaction on denaturation, acquire one of peculiar intensity when afterwards treated with GSH in high concentrations. The amount of the reactive SH group thus established in the protein complex varies with the nature of the protein as will be immediately shown. It seems to be a definite characteristic of each individual protein.

In every case the group when established has the properties described for it in the case of the muscle proteins. That its appearance is due to a reduction in the protein complex, and not to any form of adsorption of the thiol compounds used to produce it, can be demonstrated on the lines already described. It is highly resistant to oxidation by molecular oxygen in the absence of glutathione or its equivalent, whilst it is oxidised by G_2S_2 (and of course reduces the latter) under the conditions defined above.

The establishment of the SH group in its molecule determines in every susceptible protein a capacity to take up oxygen in amount which, if not high in a chemical sense, is high in relation to the phenomena which are under review. It is indeed not yet sure what are the limits of the oxidation involved.

Experiment has shown that, as already stated, the maximal production of the SH group on reduction varies from protein to protein, but the data obtained have not yet been sufficiently controlled to give them a strictly quantitative value. The matter is under study. To take one example: after treating coagula from (*a*) mixed serum proteins wholly free from lipoids, (*b*) pure crystalline egg-albumin, and (*c*) crude fibrin, with $M/10$ solutions of GSH or thioglycollic acid, I found that the oxygen equivalent of the SH group established in (*a*) (determined as described above) was of the order of 300 mm.^{3} per g. protein, of that in (*b*) 160 mm.^{3}, and of that in (*c*) at most 60 mm.^{3}. It is perhaps not sure that really pure fibrin displays the phenomenon at all.

Gelatin is incapable of developing an SH group. No protein or protein preparation which fails to exhibit a nitroprusside reaction shows any tendency to reduce solutions of G_2S_2.

The oxygen uptake of the reduced proteins in the presence of glutathione at p_H 7·6, while more or less proportionate to the amount of SH established, is, as already stated, greatly in excess of the actual oxygen equivalent of this. The specimen of mixed serum proteins just referred to, prepared free from lipoids by the ice-cold alcohol method of Hardy and Gardiner [1910] was exposed in powdered form to strong solutions of reduced glutathione and thioglycollic acid, respectively ($M/5$ in each case). Each portion was then washed with water till no trace of nitroprusside reaction was given by the washings (whenever thioglycollic acid is used this process is prolonged) and dried in a vacuum desiccator.

The oxygen uptake of each portion in the presence of G_2S_2 was then determined at p_H 7·6 in a Barcroft manometer; the two instruments being shaken side by side. (There was no uptake alone, and none with glutathione at p_H 3·5–4·5.) The uptake at p_H 7·6 was exactly the same in each case, suggesting that the maximal reduction had been produced. The figure obtained was 2350 mm.^{3} per g. protein. In Fig. 7 the curves of uptake in this experiment are given to show how definite is the condition established, and also to show that, when properly standardised, the Barcroft apparatus gives highly consistent results. The two curves are practically superimposable.

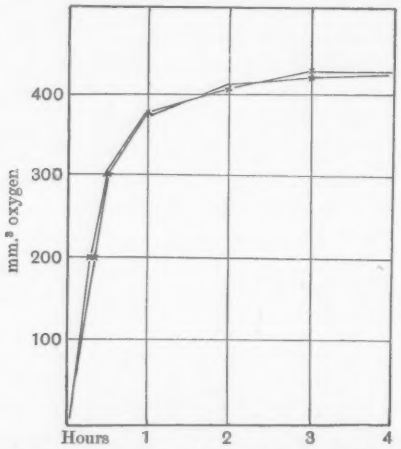

Fig. 7. Uptake of lipoid-free serum proteins (0·2 g.) after coagulation and reduction with GSH and thioglycollic acid; suspended in G_2S_2 $M/200$; p_H 7·6.

But it was easy, as in the case of the lipoid-free muscle residue, to show that the oxidation of the protein stopped at the above figure only because the fixed SH group was exhausted. Freshly reduced by GSH it showed a second uptake of nearly 2000 mm.^{3}. Reduced a third time it showed a further uptake

nearly as large, each oxidation involving, of course, the simultaneous influence of glutathione (G_2S_2) in solution. I have not yet determined what is the limit to these successive processes. If each successive uptake corresponded only to the oxygen equivalent of the SH group as originally present, or as re-constituted, the significance of the facts would be less. It is, as a matter of fact, at least ten times as great. If therefore it should prove that the processes of alternate reduction and oxidation can be yet further extended, the final amount of oxidation involved must be great enough to produce an effect upon the protein such as should be easily detected. This matter is being fully investigated[1].

One other aspect of these curious relations must be emphasised. In order that the various proteins should be reduced by contact with thiol solutions they must be in the form of a solid phase. If, *e.g.*, native serum be mixed with a high concentration of GSH and allowed to stand anaerobically, and if after some hours the mixture be diluted, the proteins being then coagulated by heat, filtered off, and quickly washed, the coagulum shows no trace of a nitroprusside reaction. None, moreover, is shown by the precipitate obtained by saturation of the mixture with ammonium sulphate, if it be first thoroughly washed by a saturated solution of the salt. The most probable explanation of this circumstance is that in the process of coagulation a molecular change occurs which is necessary to establish the group which is susceptible of reduction. If this be so, it may appear to reduce the biological significance of the facts. It must be remembered, however, that the proteins which are left in muscle after thorough washing and which constitute an important part of the permanent structure of the fibres, are susceptible of reduction in their native state. If muscle after simple extraction with water be aerated in the presence of G_2S_2 at p_H 7·4 it may be completely oxidised and deprived of its nitroprusside reaction. This is then normally restored by exposure to GSH solutions. It would be interesting to find that the less soluble among tissue proteins share some special character with blood-proteins rendered insoluble by denaturation.

VI. Factors Concerned in the Oxidations Described.

In Section III evidence was advanced to show that in the oxidation of fatty acids by glutathione the reaction changed in type with change of hydrogen ion concentration. In acid systems the thiol group acts essentially as an oxygen carrier, in neutral or alkaline systems its own oxidation involves the fatty acids in a coupled or induced reaction. Does the oxidation of protein conform to either of these types, or is it rather a process to be conveniently viewed from the standpoint of Wieland as one of hydrogen activation and transport? Some facts seemed to suggest this, but others offer difficulties. It must indeed be admitted that the ascertained facts are insufficient to make clear the nature of the phenomena.

[1] Mr A. Carruthers has found that as the result of four successive reductions and oxidations a sample of lipoid-free serum proteins took up 10 cc. per g. The limit was not then reached however.

In discussing the evidence it will be convenient to deal first with systems consisting solely of protein and the oxidised or disulphide form of glutathione. Only proteins such as those of washed muscle which naturally contain an SH group, or denaturated proteins upon which an SH group has been conferred, suffer oxidation in such systems. It will be convenient to speak of these—for lack of a better term—as "reduced" proteins.

For a sustained oxygen uptake by unsaturated fatty acids, the conditions in the system must, as we have seen, be such as secure stability of the SH group (Section III). For the oxidation of protein, on the other hand, the conditions must favour the autoxidation of SH (Section IV). This distinction is fundamental. In each case the hydrogen ion concentration exerts a dominant action in determining these requisite conditions. In the latter case the presence of dissolved iron in minute amounts may be a necessary factor.

As the disulphide form of glutathione is reducible by the "fixed SH" throughout a wide range of hydrogen ion concentration, fat is oxidised in the presence of "reduced" protein at p_H 3·5, even though G_2S_2, and not GSH, is initially supplied to the system. Experiments in proof of this will be described immediately. On the other hand, in systems containing reduced protein and G_2S_2 alone, there is no oxygen uptake at such a grade of acidity. The disulphide in solution is reduced until equilibrium is established with the SH in the solid protein, and the system then becomes inactive, exactly as though it were under anaerobic conditions. When the p_H is changed to 7·4–7·6, the oxygen uptake already described actively proceeds. It has been shown that this uptake depends upon the existence of the fixed SH group, and also that the total uptake is much in excess of the oxygen equivalent of this.

A significant circumstance in the behaviour of a system consisting of reduced protein (or a thermostable muscle residue) and G_2S_2 is that throughout the course of its oxygen uptake at p_H 7·6 the concentration of SH in solution remains very low. If the process be arrested at successive periods during the course of the uptake and a nitroprusside test applied to the contents of the flask of the Barcroft apparatus, the colour reaction in the protein is found to diminish progressively, whilst that in the solution is very faint throughout, even when the oxygen uptake is proceeding at its highest velocity. By the time, however, the nitroprusside reaction has become negative in both, the uptake ceases. The reason for the low concentration of SH in solution is clear. Compared with the rate of the oxidation of GSH at p_H 7·6 the reduction of G_2S_2 is a slow reaction. Conditions in the system adjust therefore to this relation. Oxidation follows reduction and the process is continuous, but because of the velocity relations the intermediate reduction product never reaches more than a very low concentration. The reduction process is much less affected by p_H than is the oxidation [Tunnicliffe, 1925].

Now although the oxidation of protein by glutathione is so entirely inhibited at grades of acidity such as correspond to a p_H of 3·5–4·0, in the region of p_H 6·5–6·8 it proceeds, though at a velocity somewhat less than at 7·6.

In accordance with the relations indicated the SH concentration in solution is higher during the course of the oxygen uptake in the slightly acid system. At such a grade of acidity the rate of reduction is scarcely affected; that of oxidation is considerably slower.

All these facts seemed to justify the view that in the system now under discussion glutathione is an intermediate hydrogen acceptor. It would seem to share in a process of hydrogen transport of the kind pictured by Wieland and Thunberg. On these lines the smallness of its concentration in an active system offers no difficulties. It is indeed, as was indicated above, to be expected. This was the view put forward in previous communications in respect of the oxidation of washed muscle, or its thermostable residue, by glutathione. I had myself always pictured that there were constituents of the washed muscle oxidised on such lines; constituents, namely, which are capable of reducing the disulphide form; an essential for their oxidation on the lines conceived. It would now seem from evidence discussed in the last section, that the one essential reducing factor is the SH group present in the insoluble muscle proteins. When limited to this factor the above view certainly holds. But what is the mechanism of the oxidation in protein which, while certainly depending upon the presence of the fixed SH group, involves an uptake of oxygen ten times as great as that required to oxidise the SH group itself? It seemed during earlier stages of this enquiry as though the behaviour of the SH group in the muscle residue might only be, so to speak, an indicator, and that other agencies in the tissue also possessed reducing power. But when it was realised that the material oxidised was protein, it was further found that no protein reduces G_2S_2 unless it contains an SH group. Moreover, as was stated in the last section, Tunnicliffe's quantitative studies, and data which I have since repeatedly obtained myself, show that when the muscle residue (or a "reduced" protein) is in contact under anaerobic conditions with solutions of G_2S_2, the maximal reduction of the latter corresponds quantitatively with the reducing capacity of the fixed SH group. Nevertheless, aerobically, the oxygen uptake is ten-fold the oxygen equivalent of this. These results seem to preclude the possibility that the whole process of oxidation is one of hydrogen transport. Yet if oxidation, say at unsaturated linkages in the protein molecule, or the oxidation of structures other than the SH group, depends upon a function of SH in solution (akin, say, to that displayed in the oxidation of fatty acids) it is difficult to understand how it actively proceeds when the concentration of soluble SH in the system is so exceedingly low.

Faced with this difficulty it is perhaps legitimate to consider the following possibility, even if it be unlikely. In the process which results in the oxidation of protein by glutathione, the fixed sulphur group might be an additional intermediate hydrogen acceptor. While the SH in the protein reduces G_2S_2 in solution, it might be necessary, in order that the process should continue, that the fixed SH group should itself be again reduced by other factors in the protein which, while ultimately susceptible of oxidation, cannot directly reduce

G_2S_2. Now, the latter reduction may be a much slower process than the former, and may not have been given time to display its effects appreciably in the anaerobic experiments described in the last section. Some of these were prolonged, however, and the suggestion is unlikely. Yet the following facts should be considered here. If washed muscle be thoroughly aerated in the presence of glutathione until it gives no trace of a nitroprusside reaction, it will upon heating in water to 100° again give a reaction which is quite intense, and it will now again reduce G_2S_2 though, quantitatively, much less effectively than when it has itself been reduced by contact with a thiol compound. The proteins behave like egg-albumin. This shows at least that the reconstitution of the fixed SH group may in some sense result from changes occurring within the solid (protein) phase of the system. But that this reappearance of the SH group does not strictly represent the process postulated above (accelerated by the higher temperature) is indicated by the fact that though it occurs immediately at 100°, and more slowly at 60°, it does not seem to occur, even slowly, at temperatures below the coagulation point of the muscle proteins. It involves denaturation, therefore. But, as was made clear at the close of the last section, the proteins of washed muscle do not need to be denaturated in order to show their normal large oxygen uptake. It is difficult therefore to see how the whole process of protein oxidation by glutathione can be entirely one of hydrogen transport. Provisionally at least, it seems we must look to the SH group in solution as the promoter of oxidation, maintained, as it is, in very low concentration, by the slow reducing action of the fixed SH group.

If, however, GSH be supplied to the system initially (instead of G_2S_2) there is no increase in the oxidation of the protein. What happens is noteworthy. If two Barcroft manometers be shaken side by side, each containing muscle powder and glutathione in Ringer's solution, but with GSH in one and G_2S_2 in the other (all concentrations and conditions being otherwise the same) curves of uptake such as those in Fig. 8 are (at p_H 7·6) consistently obtained.

The system containing GSH shows the higher initial velocity, but this must be due in the main to the independent oxidation of SH. For at a point when the excess of oxygen taken up by this system has become nearly (but not quite) equal to that required for the oxidation of all the GSH added, the two curves become parallel for the rest of the period of uptake. During this later period, which may be prolonged, each system contains the same exceedingly low concentration of SH in solution. This means that in both cases the oxidation of the protein is essentially dependent upon the fixed SH group as the agent which reduces glutathione. The reduction of the S-S form is, as we have seen, a slower reaction than the oxidation of the SH form and therefore finally determines the velocity of oxygen uptake.

Having dealt with the oxidation of fats and proteins separately, a few words may now be said concerning the behaviour of the original thermostable residue from muscle as used by Dixon and myself and afterwards by Meyerhof. Its oxygen uptake has received preliminary discussion in Section III. It is chiefly of interest from the fact that it contains both protein and fat, so that

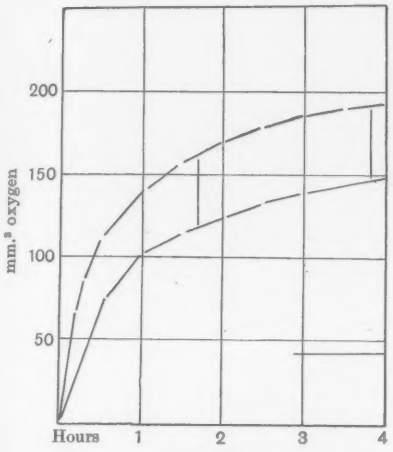

Fig. 8. Uptake of lipoid-free muscle powder at p_H 7·6. Upper curve with GSH, lower curve with G_2S_2. The horizontal line by its position on the scale indicates the oxygen equivalent of the SH initially present in the former system, and the length of the vertical lines between the curves corresponds with this value.

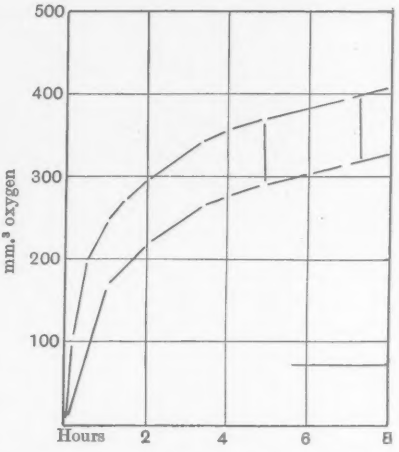

Fig. 9. Uptake of thermostable muscle powder (0·2 g.) at p_H 7·6. Upper curve with GSH, lower curve with G_2S_2. Indications as in Fig 8.

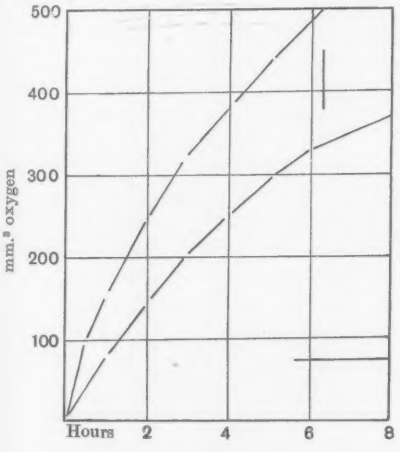

Fig. 10. Thermostable muscle powder (0·2 g.) at p_H 6·0. Upper curve GSH, lower curve G_2S_2. Horizontal and vertical lines show oxygen equivalent of GSH. See Fig. 8.

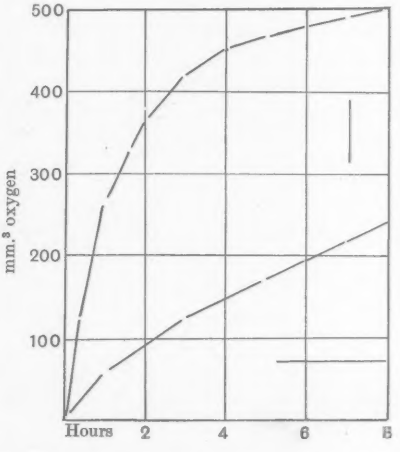

Fig. 11. Thermostable muscle powder (0·2 g.) at p_H 3·8. Upper curve GSH, lower curve G_2S_2. Horizontal and vertical lines show oxygen equivalent of SH.

its uptake varies with changes in hydrogen ion concentration on lines that are instructive. It has been shown in previous sections that at p_H 3·5–4·0 fatty acids are continuously oxidised during the survival of the SH group, while no oxidation occurs in protein. At p_H 7·4–7·6 conditions present secure the peculiar oxidation of protein which has been so fully discussed, while fatty acids undergo only a limited oxidation. With change of p_H therefore different materials predominate in the oxidation and the total uptake may not be the same even with the same preparation. Variation in the fat content of preparations will also have a different influence at different hydrogen ion concentrations. These relations will be sufficiently illustrated by the curves of Figs. 9, 10 and 11, which show the course of oxygen uptake by a muscle powder at p_H 3·8, 6·0 and 7·6 respectively. In each case, the effects of adding GSH and G_2S_2 initially are compared.

The total concentration of glutathione was the same in each system. In one of each pair as compared the dipeptide supplied was partially (but not completely) reduced. The actual oxygen equivalent of the SH present initially is indicated in the figures.

At p_H 7·6 (Fig. 9) the curves are not different in type from those yielded by a lipoid-free preparation (Fig. 8) though the total uptake may be higher. It will be seen that after showing a more rapid initial rise, the curve of uptake with GSH becomes parallel to that with G_2S_2. The oxidation mainly concerns protein, and its velocity in each case is ultimately controlled by the rate of reduction of G_2S_2 by the fixed SH group (see earlier discussion). Hence the remarkably accurate parallelism which is established after the excess of GSH has been oxidised in the system which contained it initially.

At p_H 6·0 (Fig. 10) both fat and protein are oxidised in each system. The curves do not become parallel. In the system which initially contained GSH, an efficient concentration of the SH group is longer maintained and therefore more fat is oxidised than at 7·6. In that supplied with G_2S_2 the uptake depends as before upon the velocity of reduction, but in this more acid medium the SH concentration is again maintained at a higher level, and more fat is oxidised than in the alkaline system.

At p_H 3·8 the curves diverge still more widely. Fat alone is oxidised in either system. In the GSH system the thiol group is stable and fat is more efficiently oxidised than at p_H 6·0. In the G_2S_2 system the oxidation of the fat by SH is controlled by the slow reduction process[1].

These brief references to the curves will be sufficient to indicate the behaviour of systems which contain both "reduced" proteins and fats. The most significant aspect of their behaviour is seen in the influence of protein in determining the oxidation of fat in circumstances which do not secure the oxidation of the latter when it is alone; for instance, when the disulphide is

[1] The experiments yielding these curves have shown how entirely the total oxygen uptake of the thermostable residue (or of water-extracted muscle) depends upon circumstances (see Section III).

supplied to the system. Without reduction of this there is no oxygen uptake; and fats do not reduce it. But if the system, in addition to the disulphide, contains a muscle residue, or any protein containing the SH group, fat is freely oxidised. This has just been shown in the case of the thermostable muscle residue (Fig. 11, lower curve). The effect is more strikingly demonstrated when excess of fat, in the form of an emulsion, is added to a system consisting of the muscle residue, or a "reduced protein," together with G_2S_2. At p_H 3·0–4·0 a large excess of oxygen is taken up by such a system as the result of adding free linolenic acid, linoleic acid or lecithin, the curve of uptake being linear if the lipoid be in sufficient excess. The velocity, however, is controlled by the relatively slow reduction process. At p_H 7·6 when lecithin or a fatty acid is added to the same system there is an uptake greater than that displayed without them; but the excess is relatively small. This would be expected if the results of Section III are taken into consideration. On the other hand, the addition of glycerides (linseed oil) to the alkaline system results in that linear uptake which results when GSH alone is added to an emulsion. It continues, as in the latter case, with an existing concentration of SH which is exceedingly small (see p. 797). Fig. 12 shows the relation just discussed.

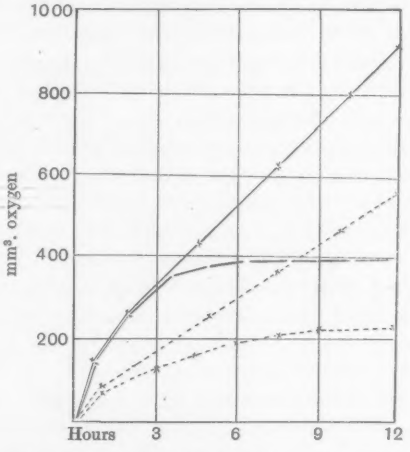

Fig. 12. Curves with continuous line show uptake of a thermostable muscle powder (0·2 g.) plus G_2S_2 with and without the addition of linseed oil; p_H 7·6. Dotted line curves, the uptake of a similar system with and without added lecithin but at p_H 3·5. Each pair of curves was obtained by the use of two Barcroft manometers shaken side by side.

General Discussion.

The experimental studies described in this paper have yielded certain results which were unexpected. Some of the phenomena indeed seem to find no close analogy in the behaviour of chemical systems which are more familiar. It is somewhat surprising, moreover, to find, when a substance presumed to promote biological oxidations in muscle is in question, that its activity should

be more easily demonstrable in the case of fats and proteins than in that of carbohydrates or their derivatives. This may be of physiological significance; but it may be rather an accidental result of the particular experimental conditions which have been empirically chosen. The influence of glutathione in promoting oxidations, has, even in cases where its potency has been already demonstrated, shown itself highly sensitive to conditions, and the effect of these has not been by any means fully explored. The highly artificial preparations of the tissue which, in pioneer studies, have been perforce and justifiably employed, are, of course, without many factors which, in the living tissue, may exert a directive influence. One illustration may be mentioned. Miss Thurlow [1925] has shown that the oxidation of the SH group when it occurs in the presence of a peroxidase may induce other oxidations (*e.g.* of nitrites) which do not occur in the absence of this latter factor. The possibilities suggested by this observation are being explored in this laboratory but as the work is being done by others the results are not discussed in this paper.

It will serve the purpose of a brief general discussion of the results obtained in the present research if a comparison be made with those described by Meyerhof in his classical paper on the subject [1923].

Meyerhof was the first to observe the oxidation of lipoids (lecithin, linolenic acid) under the influence of a thiol compound (thioglycollic acid). He was inclined to think that this oxidation might be the only one induced by glutathione in muscle, though in the closing words of the paper just referred to he disavows any finality in that opinion.

He found that the oxidation of the lipoids occurred in acid systems (most effectively at p_{H} 3·0–4·0) and upon the behaviour of such systems he based his view as to the mechanism of the oxidation by SH. He did not observe the oxidation of fats (limited but quite significant) that occurs at hydrogen ion concentrations nearer to that of living tissues (Section IV). Nor did he observe the oxidation of protein which has been described in this paper. In an earlier publication [1918] he was inclined, it is true, to attribute to the oxidation of protein part of the uptake of oxygen displayed by bouillon to which thioglycollic acid had been added. In his later work [1923], however, he rejected this view on finding that serum proteins when thoroughly freed from lipoids showed no uptake. This observation was undoubtedly correct. The native blood proteins show no uptake whatever under the influence of glutathione; but this, as will be understood from facts advanced in previous sections, is because they lack what has been termed throughout this paper "a fixed SH" group. It is of interest indeed to note that the proteins of the fluids of the body, and the albuminoids of connective tissues generally, are immune from the type of oxidation suffered by the proteins of such an active tissue as muscle. They are not exposed to the influence of glutathione, which appears to be absent from the plasma, nor are they exposed to influences which establish in them the second factor necessary for the oxidation in question, namely, the fixed SH group.

But Meyerhof also found that muscle preparations when deprived of all lipoids showed no more than a slight and non-significant oxygen uptake with thioglycollic acid or cysteine. He could not therefore attribute an oxidation to muscle proteins, whereas, as has been shown in previous sections, their oxidation by glutathione is a definite and quantitatively important phenomenon.

The difference between Meyerhof's results and my own is not difficult to explain, however. In the first place the proteins are not oxidised at all in acid systems. Now although it happens that in reporting the results obtained with alcohol-ether extracted preparations [1923, Table IX, p. 542] he does not specify the p_H employed, it is probable, from what comes earlier in his paper, that the systems studied by him were acid. If so, his negative results are fully explained. There is probably a further reason for them, however. If the facts have been made clear in the discussion of Sections V*a* and VI it will be understood that the oxidation of proteins by glutathione unlike that of fats in acid systems, depends upon a succession of oxidation and reduction. That the SH group in solution should be progressively oxidised is essential; but when in any system containing a fat-free muscle residue (or any "reduced" protein) is supplied with SH in excess of a certain low concentration, the excess is (at p_H 7·6) rapidly and (with respect to the protein) ineffectively oxidised. Only when the oxidation adjusts to a slower reduction process; only that is when the properties of the fixed SH group control the velocity of change (Section VI, p. 809) is the effective mechanism for protein oxidation established. Now I have found that neither thioglycollic acid (iron-free) nor cysteine is so efficient as glutathione in establishing such a system. Those who are familiar with Meyerhof's paper will remember that his own experimental studies finally led him to attach a predominant importance to the function of the SH group as an oxygen carrier (being especially active therefore in systems where its concentration is maintained) rather than to the circumstance that it is a reducing agent easily oxidisable to an S-S group, from which it is again easily reproduced by reduction. It was these properties which determined my own previously published views as to the importance of glutathione as a carrier of hydrogen. Nothing, I think, is now more sure than that both views may be correct. With change of conditions glutathione from being an oxygen carrier (oxidation of fatty acids in acid systems) may become, to use a term which is at least convenient, a carrier of hydrogen (oxidation of proteins in neutral or slightly alkaline systems). It is true that, in protein, only the fixed SH group has so far been proven to be a direct source of this transportable hydrogen. The large uptake of oxygen (many times the oxygen equivalent of the above group) which a "reduced" protein displays occurs on lines which are not yet clear. It is at any rate certain that it proceeds only for so long as the fixed SH group survives. Moreover, in any case the influence of this simple reducing factor by itself secures exactly the result which has been pictured [Hopkins, 1923] as following from the ability of tissue agencies to reduce glutathione in

solution. It links one form of oxidation to another; *e.g.* that of proteins to that of fats. It is an essential controlling agency. In neutral or alkaline systems the thiol form of glutathione is so rapidly oxidised that, in the absence of a reducing factor, its disappearance quickly renders the system inert. The slow reducing action, for which the fixed SH group is responsible, maintains a concentration of SH in solution, and determines the whole velocity of oxidation. The process continues so long as (and only so long as) the SH in the protein survives. In acid systems its reducing power remains, and, by again securing a continued concentration of SH, determines a prolongation of the oxidising capacity of the system (Section VI). In the cell reduced proteins always co-exist with glutathione. Their coexistence and their mutual influence may well prove to be a biological essential. It must only be remembered that experimental studies have so far been confined to a preliminary analysis of oxidising mechanisms. We have no knowledge of their complex relations in the tissues. In a tissue, for instance, at least after it has been removed from the body and prepared for examination (by which time it has, of course, been in an essentially anaerobic condition for an appreciable interval), the whole of the glutathione is in the reduced form [Tunnicliffe, 1925]. I have indeed found that a tissue in such circumstances—the liver at any rate—will rapidly reduce considerable quantities of additional glutathione when it is added in the disulphide form.

I am well aware indeed that on first acquaintance the curious and at many points obscure phenomena described in this paper may seem to lack biological reality. The experimental results described, however, are constant and easily reproducible. They are yielded by systems which contain actual cell constituents in contact with one another, and depend upon actual properties of these constituents. Such studies would seem to be a necessary, if remote, antecedent to a fuller understanding of the behaviour of the same constituents in the organised phenomena of the living cell itself.

Summary.

In acid systems (p_H 3·0–4·5) glutathione (GSH) promotes the oxidation of certain unsaturated fatty acids and of lecithin. The mechanism of oxidation is, in this case, probably that postulated by Meyerhof [1923].

In neutral or alkaline systems (p_H 7·4–7·6) the nature of the process is different. During the oxidation of the SH group fatty acids are simultaneously oxidised in such a way that equipartition of oxygen occurs.

The oxidation of the glycerides of the above fatty acids proceeds under the influence of GSH, but in detail the process displays remarkable differences from the course of oxidation in the case of the free acids.

Glutathione (GSH or G_2S_2) promotes an oxidation of certain pure proteins on peculiar lines. The oxidation proceeds only when and while the protein itself displays (as shown by the nitroprusside reaction) an SH group. The

proteins of water-extracted muscle contain this group, and suffer the oxidation in question in neutral or faintly alkaline solutions. In acid systems (p_H 3·0–4·5) no oxidation of protein occurs.

In this oxidation the protein SH is oxidised and the nitroprusside reaction disappears, while the total oxygen taken up amounts to ten times the oxygen equivalent of the SH. The protein can then be again reduced; the SH group reappears and (with glutathione) a further uptake of oxygen becomes possible, which is again greatly in excess of the oxygen equivalent of the SH. Successive reductions and oxidations of this kind can be carried out, till finally the protein takes up at least 10 cc. per g. and possibly much more.

At any stage the "oxidised" protein (showing no nitroprusside reaction) can be reduced by contact (as a solid phase) with strong solutions of glutathione (GSH), cysteine, or thioglycollic acid, so that it again displays an intense nitroprusside reaction. Such a "reduced" protein then again takes up oxygen on the lines already described. When insoluble protein is in contact with solutions of glutathione, or other thiol compounds, equilibria are established between sulphur groupings in the solid (protein) phase and sulphur groups in solution. Mutual oxidation and reduction occur as reversible processes.

Whilst the proteins of water-extracted muscle undergo oxidation and reduction, on the lines described, in their native state, certain other proteins (those of blood serum, for example) need to be first denaturated. In an insoluble form the proteins of serum can be so reduced by contact with strong solutions of thiol compounds as to display an intense nitroprusside reaction. They then behave like the muscle proteins.

I owe much to the experimental skill of my assistant, E. J. Morgan. His help is provided by the Medical Research Council and to that body my grateful thanks are due.

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CXXII. ANTINEURITIC YEAST CONCENTRATES. I.

By HAROLD WULFF KINNERSLEY
and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

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The present communication[1] deals with active curative preparations from yeast which have been obtained by the use of polyneuritic pigeons as test animals. The outstanding problems in connection with vitamin B are firstly, concentration of the vitamin, and secondly, whether the properties ascribed to vitamin B reside in one or more constituents. In order to avoid begging the question the word "torulin" is used throughout [Edie, Evans, Moore, Simpson and Webster, 1912] to mean the principle in yeast which cures symptoms of head retraction in pigeons induced by feeding upon polished rice.

Technique.

The technique described previously has been used [Peters, 1924]. Test samples have been introduced into the crop. The pigeons have not been fed artificially. Only birds conforming to the conditions previously laid down have been used for the tests. Particular care has been taken to exclude cases of the illusory heat cures, to which allusion was made before. Since then much evidence has accumulated. Birds which happen to get convulsions in the outside aviary must be left for a clear 3 hours before treatment after bringing into the warmer laboratory air. In many cases such birds clear up completely, and do not show symptoms again for a period of 1–3 days. Three days seems to be the limit for these heat cures. We have no explanation to offer for this interesting fact which has been investigated by Roche [1925]. Excluding cases of heat cure there are still found birds which do not fall into the ordinary categories, so that for tests upon important material, it is essential to use more than one bird for standardisation.

Measurement of activity.

We have standardised our results by the use of a curative and protective test, by means of the following equation,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

[1] A preliminary account of this work appeared in *J. Soc. Chem. Ind.* (1925), 531.

Dry weight here means the weight obtained by drying at 100–105° for 1 hour, after subtraction of the weight of ash. Thus if 4 mg. of a preparation cure and protect for 4 days, the activity is considered to be 100 T.A. Use of this equation implies at least two assumptions: (1) that there are not wide variations in the amount of torulin required by different pigeons, and (2) that no antitorulin factors are present. With regard to (1) it is likely that account must be taken ultimately of both weight and temperature to which pigeons have been exposed. The average weight of the pigeons used by us at the time of convulsions is 270 ± 40 g. Young birds have not been employed. (2) is at present a pure assumption. In practice we have found that the results obtained for times of cure between 2 and 10 days are reasonably quantitative, so much so that it has been possible to follow the distribution of torulin between various fractions with ease. After 10 days the results are not reliable. An example of what is meant is given in Table I.

Table I.

Pigeon	cc. given	Cure and protection (days)
1	0·2	10
2	0·2	9 (after treatment with nitrite)
3	0·2	10
4	0·5	19

Including the nitrite-treated preparation bird 2, 0·2 cc. of this preparation gave in days 10, 10, 9. Upon this basis 0·5 cc. should protect for 25 days, whereas protection only lasted for 19 days.

Table II deals with tests upon an active preparation. It shows what accuracy may be expected from such quantitative tests, and also certain limitations in its use.

Table II.

Pigeon	Dose (cc.)	Cure and protection (days)	Day dose (mg.)
5	0·1	2·5	0·068
6	0·1	2·0	0·085
	0·2	3·0	0·11
7	0·2	4·0	0·085
8	0·3	6·0	0·085
9	0·3	7·5	0·068*
10	0·3	13·0	0·025
11	0·24	2·8	0·24

* This preparation was dried at 100° before administration and found to weigh 0·51 mg. The average for pigeons 5–9 is 0·084 mg. per day, 1190 T.A.

It will be seen that there are three groups: (*a*) the majority of birds 5–9, which behave well to a standard, (*b*) bird 10 which required much less than the standard, and (*c*) bird 11 which required much more. Bird 10 has been omitted, and represents an abnormality in response, which appears occasionally. It is possible that such birds still have sufficient torulin left in their system, and that the small dose given is enough to set free this store. Bird 11 has also been omitted, for the reason that it was not in good condition when the dose was given, and was also a bird which had been subjected to the tests

over a period of 5 months in the laboratory. It must be admitted that at present it is not possible to eliminate the element of judgment in the selection of suitable cases. Use of the equation is however justified by the fact that by its aid we have obtained torulin concentrates some 200 times as concentrated as in the charcoal concentrates. During the progress of the research we have been increasingly impressed by the quantitative response of the majority of test birds to the fractions given.

Experimental.

It is not proposed to give at length the numerous curative experiments made in the course of fractionation. In each case, before proceeding to a further step, results have been checked by the use of birds. A few general statements may be made as to procedures which have not been helpful, when applied to the charcoal concentrates. With phosphotungstic acid it was not found possible by a varied extraction to recover the active principle in reasonable amount. At this stage also adsorption upon kaolin, or upon specimens of fuller's earth procurable by us proved valueless, as did also precipitation with lead acetate in alkaline solution. In the latter case all the torulin present was recovered from the filtrate. We have not been successful in preparing from these concentrates active picrates similar to those described by Seidell [1924].

The following method we have found successful, when working upon a scale of some 7–21 lb., using D.C.L. baker's yeast.

Up to the stage involving the use of acid alcohol to extract torulin from the charcoal, the technique has been the same as previously described [Peters, 1924], with the exception that in later preparations the treatment with BaS has been omitted. Instead charcoal adsorption has been applied direct to the acid fluid after removal of the mercury precipitate. After removal of the first amount of charcoal, the fluid has been neutralised to methyl red and again treated with charcoal in similar amount. The charcoal residues are combined after washing with distilled water, and concentrated. Further treatment can be then carried out as described below. Unless otherwise stated, all precipitates have been removed by the centrifuge and washed with appropriate solution. Concentration has been carried out *in vacuo* under the water pump with a temperature not exceeding 60° in the apparatus described [Peters, 1924].

Stage 1. The charcoal concentrate was diluted with a convenient amount of water and alcohol added to a concentration of 60 %, the precipitate removed and discarded, and the centrifugate (cf.) concentrated to remove alcohol.

Stage 2. The concentrate from stage 1 was diluted with water and 25 % lead acetate added until precipitation was complete, the precipitate removed, the centrifugate and washings combined, treated with HCl to remove excess of lead and concentrated. Any precipitate appearing during concentration can be discarded.

Stage 3. The acid concentrate from stage 2 was treated with methyl alcohol to a concentration of at least 90 % (more does not matter), the

precipitate removed, and the centrifugate concentrated to remove methyl alcohol.

Stage 4. The solution from stage 3 was made up to a convenient volume with water and treated with "dialysed iron" 10 %, about 7 cc. being required for a 7 lb. sample. The precipitate was removed by the centrifuge, and washed. This washing was found to be important. Normal NaOH, about 10 cc., was then added until a precipitate ceased to come down, at which stage the fluid is very alkaline. Care must be taken to carry this stage through quickly, and not to allow the solutions to rise above room temperature. We have found in confirmation of the work of others that torulin is inactivated by heating with NaOH. The heavy precipitate is removed and washed with dilute caustic soda. The centrifugate was made acid to litmus as soon as possible and concentrated.

Stage 5. Ethyl alcohol was then added to a concentration of 85 %, the precipitate removed and washed with 85 % alcohol, and the centrifugate concentrated. Alcohol was then added to 95 % and the process repeated. It is best to re-dissolve the gummy precipitates obtained in a minimum amount of water and throw out again with alcohol at this stage, at which there is liable to be loss.

Stage 6. The 90 % alcoholic solution was treated with half a volume of ether, the precipitate removed, taken up in a minimum of water, made up to 90 % alcohol with absolute alcohol, and re-precipitated with ether. The combined centrifugate from the two precipitations was concentrated as far as possible, the extract dissolved in a minimum of water, and made up to a strength of 95 % alcohol with absolute alcohol, any precipitate appearing at this stage being removed.

Stage 7. The fraction obtained was then gradually worked up to higher concentrations of alcohol, the object being to obtain the fraction soluble practically in absolute alcohol. When the fraction soluble in about 99 % alcohol is obtained it is well to take it to dryness, and then treat with absolute alcohol, and allow to stand out of contact with air overnight. About 40 cc. of absolute alcohol were used at this stage for a 7 lb. preparation. The precipitate can then be re-extracted with fresh alcohol. The torulin will be found in the absolute alcohol solution. The final stages require care, and it is well to check the absence of torulin from the final precipitates. The material at this stage is apt to vary. In the case of four preparations which have been worked up to this stage, the activity of the final product has varied between 50–100 T.A. A yield of some 2800 day doses may be expected from 21 lb. of yeast.

Owing to the variability of material, we do not claim that the method described is more than empirical. We have found in working upon the larger scale that it is necessary to repeat stages in order to get these final fractions soluble in alcohol and free from gum. The advantage of separation from solvents lies in the freedom from ash and gum of the final fraction, and the ease with which a false step may be retrieved.

Further treatment of the concentrates.

By fractionation of a product of 63 T.A. in mixtures of ether and alcohol in which the amount of water was reduced to a minimum three fractions were obtained: (*a*) a gummy fraction thrown out of the alcohol by the addition of mere traces of ether, (*b*) an intermediate fraction thrown out by ether up to four volumes of ether to one of alcohol, and (*c*) a fraction soluble in ether-alcohol. The latter contained an oil, comparatively insoluble in water. Fractions (*a*) and (*c*) were relatively inactive. The intermediate fraction when treated with silver sulphate in acid solution threw down an inactive precipitate. From the centrifugate after treatment with silver nitrite in the presence of HCl, ammoniacal silver hydroxide threw down a precipitate, which was extracted with HCl and alcohol, and yielded the preparation of 1190 T.A., tests upon which are described in Table II. This contained ammonium chloride as an impurity, and some evidence has already been obtained that the activity may be pushed to 3000 T.A. This being so, it does not seem unlikely that the activity of torulin itself may prove to lie between 10,000 and 100,000 T.A. or in the region of 0·001 mg. per day for the normal pigeon. This would only bring it into line with substances like pituitrin.

Weight maintenance activity.

It is difficult to know how to relate our preparations to those described by Seidell [1921, 1924] and Emmett and Peacock [1925], both of whom have employed weight maintenance tests. The latter observers describe a preparation, from a source unmentioned, capable of maintaining the weight of pigeons fed upon polished rice in doses of 0·1 mg. per day and of causing gain of weight in doses of 0·15 mg. per day. For the sake of some comparison with the weight maintenance tests, we have carried out the following experiments 1 and 2, of which the details are recorded in Figs. 1 and 2. Exp. 1 was designed to constitute a severe test for protection against polyneuritis, and Exp. 2 to place the bird under a condition in which it should easily show a gain in weight.

Exp. 1 (Fig. 1). Two pigeons upon a polished rice diet were kept outside in the winter weather. For the first 16 days the diet was polished rice alone; following this each bird received every second day 0·17 mg. of the preparation used in Table II. This should constitute a border-line amount to protect against polyneuritis at laboratory temperature. On the 32nd day, as one day was accidentally missed, four doses were given. The weight of the birds steadily fell. Bird 12 died of general weakness on the 44th day without showing symptoms of head retraction. It was found to have undigested rice in its crop. Under the conditions of the experiment the birds might be expected to get symptoms by the 30th day at latest, so that this bird may be considered to have been protected for at least 14 days. Bird 13 lasted until the 50th day, when it developed symptoms of head retraction thereby showing that

protection had been afforded previously by the preparation, but that the amount given was rather low. An extra dose of the preparation on this day cured the symptoms, but the bird died of general weakness on the next day, and was found to have no food in the crop. Doubtless not much stress can be laid upon evidence drawn from prolonged feeding upon polished rice at a stage when deficiencies other than the one under study may be present, but it seems reasonable to conclude that protection against polyneuritis may be afforded for many days under severe conditions without weight maintenance.

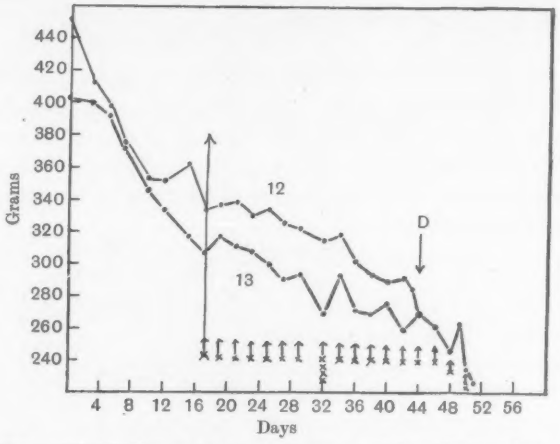

Fig. 1. Exp. 1. Pigeons 12, 13. Ordinates g. weight. Abscissae days. D = death of pigeon 12. × = 2-day dose ≡ 0·17 mg. T.A. 1190.

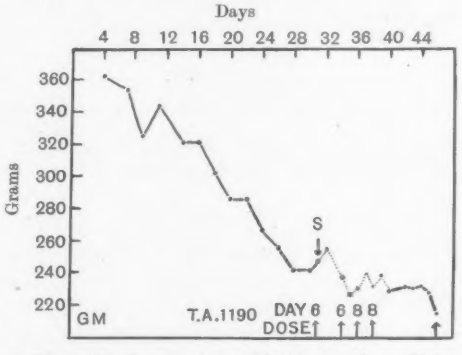

Fig. 2. Exp. 2. Pigeon 14. S = symptoms of head retraction. Diet, polished rice.

Exp. 2 (Fig. 2). During the hot summer weather, bird 14 was kept indoors until it showed symptoms of head retraction on polished rice. After this it was given several doses of the same preparation as in Exp. 1. The time at which

symptoms appear in these cases is usually later than under the conditions of Exp. 1. In this case it was the 29th day. The pigeon did not maintain weight even with 3–4 times the day dose, when conditions as regards the general rate of metabolism must have been particularly favourable.

It is clearly necessary to carry out extended tests with other preparations of this nature, but we feel that it is justifiable to conclude that highly active antineuritic preparations can be obtained which have no effect upon the weight curves in doses above the amount necessary to cure symptoms of head retraction. The evidence in this communication therefore supports the view that vitamin B consists of two factors.

Summary.

1. Heat cures must be avoided in working with pigeons suffering from symptoms of head retraction induced by feeding upon polished rice.

2. Using the word "torulin" to mean the principle in yeast curative of these symptoms, the following equation gives quantitative results in most cases, namely,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

3. A yeast concentrate has been obtained of 1190 T.A. which cures and protects in doses of 0·084 mg. per day.

4. Doses of this concentrate which protected against polyneuritis for many days did not cause increase in weight of pigeons after cure of the polyneuritic symptoms.

We wish to record our thanks to the Medical Research Council for a grant to one of us (H. W. K.), and for a grant for material for research. We are also grateful to H. Poulter for help with the yeast preparations, and to Clapton for help with the animals.

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CXXIII. RESEARCHES ON THE FERMENTATION OF DRIED TOBACCO.

I. THE METHODS FOR SEPARATING NICOTINE AND AMMONIA.

By ANDOR FODOR and ADOLF REIFENBERG.

From the Biochemical Institute of the Hebrew University, Jerusalem.

(*Received August 26th, 1925.*)

Our object in this research was to separate the nicotine from the other volatile nitrogen-containing bases contained in extracts or expressed juices from tobacco leaves, and to estimate them. In the earlier processes insufficient consideration has been given to this separation.

Kissling's method [1920] as usually carried out has the disadvantage that when extracting the leaves with ether, ammonia among other things also passes into the solvent; comparative tests made by us have shown this to be the case in a manner which places the result beyond the possibility of doubt; further, the distillate obtained by Kissling's method always gives the familiar Nessler's ammonia reaction, whilst pure nicotine gives with Nessler's reagent only a white precipitate, which on the addition of the slightest amount of ammonia becomes coloured.

Toth [Kissling, 1920, p. 98] seeks to avoid the presence of ammonia by allowing the latter to be adsorbed by gypsum, but according to Rasmussen's [Kissling, 1920, p. 99] and Schröder's [1911] investigations the Toth process gives inexact results. König extracts tobacco leaves with toluene and estimates the nicotine polarimetrically. Toth [1911], however, holds this method to be unsuitable because dark-coloured solutions are unpolarisable.

Bertrand and Javilliers [1909] precipitate the nicotine as silicotungstate, decompose this compound in a current of steam and ascertain its amount by titration. This method was unsuitable for our purposes because it did not permit of the estimation of the ammonia; moreover silicotungstic acid is an expensive reagent and difficult to obtain.

Harrison and Self [1912] estimate the ammonia and nicotine obtained by steam-distillation of tobacco after the addition of alkali, absorbing the distillate in titrated acid and then titrating back; they then precipitate the nicotine with iodine and, after destroying the colour of the iodine by means of thiosulphate, distil off the ammonia. The difference between the two titration values gives the nicotine content.

We used the following method. The nicotine, in the mixture of ammonia and nicotine obtained from tobacco leaves by direct steam-distillation, is, after making the liquid weakly acid with sulphuric acid, precipitated by means of a solution of mercuric iodide in potassium iodide (3 g. per 100 cc. water). Under these conditions, ammonia gives no precipitate. The precipitate is allowed to remain overnight and is washed by decantation; it has a wax-like crystalline character. The filtrate obtained is quite clear and free from nicotine. The precipitate can be estimated by the Kjeldahl method, whilst the ammonia can be distilled over from the filtrate in the usual way, an excess of potassium iodide being added before making alkaline to prevent the formation of a Nessler precipitate and consequent loss of ammonia.

For this series of researches it is sufficient to know the total titration value of the volatile nicotine and ammonia, and then, after precipitating the nicotine, to estimate the ammonia content of the filtrate. The difference between the two titration values gives the nicotine content.

The following figures show that this method gives satisfactory results.

We examined a control solution made up as follows:

20 cc. nicotine (pur. prep. Merck) + 20 cc. $N/10$ NH_3 + 60 cc. $N/10$ H_2SO_4.

The nicotine solution was of about 2 % concentration, 10 cc. corresponding to 13·2 cc. $N/10$ H_2SO_4.

(Here and in the following the titrations were made against sodium alizarinsulphonate as indicator.)

20 cc. of this mixture were treated in the way described above.

After distillation the filtrate gave a titre corresponding to 4·4 cc. $N/10$ H_2SO_4. The nicotine precipitate gave after a Kjeldahl determination a titre equivalent to 5 cc. $N/10$ H_2SO_4.

It was found that when one commenced with the same quantity of nicotine, without however adding ammonia, a titration value corresponding to 0·3 cc. $N/10$ H_2SO_4 was always obtained. (Each determination was repeated several times.)

After correcting the experimental value the following data emerge (cc. $N/10$ H_2SO_4):

Value	Nicotine	Ammonia
Calculated	5·3	4·0
Found experimentally	5·0	4·4
Corrected	5·3	4·1

It appears that the nicotine preparation (Merck) contains, though in very small amount, some nitrogen-containing impurities. Thus, for example, pyridine in weakly acid solution gives just the same reaction as nicotine towards KI-mercuric iodide but in contrast to it is readily soluble in excess of the precipitating agent and is therefore driven over and estimated with the ammonia.

The method reduces itself to two distillation processes:

(1) Driving over the nicotine together with the ammonia by steam-distillation.

(2) Distilling over the ammonia out of the filtrate from the nicotine mercury compound (as in the usual NH_3-distillation method).

In the application of this method to tobacco extracts there is of course the danger that the value in the first distillation may be too high, because besides nicotine and ammonia, other more or less volatile bases may pass over at the same time.

If these are also precipitated by the nicotine-precipitating agent, too high a nicotine value will be obtained, since the ammonia values (in the second distillation) will be correct.

If, however, no precipitation occurs, then conversely accurate nicotine values but too high ammonia figures are obtained (*e.g.* with pyridine).

Usually the latter will be the case, so that therefore the value, which when working with tobacco is calculated as NH_3, will generally be more correctly expressed as the value obtained for volatile non-nicotine bases.

Finally, it is to be observed that it is part of our object to find purely physical methods of separation, of practical feasibility, which will next be dealt with.

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CXXIV. RESEARCHES ON THE FERMENTATION OF DRIED TOBACCO.

II. THE ENZYMIC PRODUCTION OF VOLATILE PRODUCTS FROM NICOTINE UNDER THE INFLUENCE OF TOBACCO-LEAF EXTRACTS.

By ANDOR FODOR and ADOLF REIFENBERG.

From the Biochemical Institute of the Hebrew University, Jerusalem.

(*Received August 26th, 1925.*)

Introduction.

The decomposition of nicotine during the so-called fermentation process which takes place in the tobacco leaf is one of the most important reactions concerned in this complicated process, as a result of which the raw or dried tobacco becomes converted, in the fermentation stores, into the valuable product of commerce. According to the statements in the literature, decomposition of nicotine also occurs in green tobacco before drying. It is our intention to study the processes taking place during the fermentation, especially to find out the enzymic constituents of the cells of the tobacco leaf which take part in the fermentation. In this connection the literature is lacking in systematic investigations, although individual biochemists express the supposition that oxidases play a part in the process [Loew, 1899, 1900, 1901; Jensen, 1908]. On the other hand, many investigators to-day suggest that the process of fermentation here concerned depends upon bacterial action [Schmidt, 1924; Behrens, 1896, 1901; König, 1900; Suchsland, 1892].

The question is still open and has to be investigated whether or not bacteria, either directly or indirectly, limit the actual fermentation process.

We prepared, as will be shown below, expressed juices and macerates from tobacco leaves under the usual conditions of sterility and examined their effect on nicotine.

We found as a result that definite effects take place, and that volatile nitrogen-containing bases are produced, which can be estimated after distillation.

From their effect on silk peptone (increase of amino-nitrogen) it was ascertained that these juices are able to produce a peptolytic effect. This observation is obviously in agreement with the fact, known in the literature, that protein disappears during the fermentation process. The following researches demonstrate these results.

The problem as to what products are formed from the nicotine on its

decomposition is important. In a preliminary investigation we attempted to find whether, after allowing the nicotine to be acted upon for 2 hours at 37° by tobacco extracts, demonstrable quantities of acid are produced.

It was found that the nicotine solution used as substrate had after approximate neutralisation a p_H value of 4·79. The expressed juices, also approximately neutralised, had a p_H value of 5·74. This gives by calculation for a mixture of equal volumes a p_H of 5·05, whereas the electrometric determination gave for this value the figure 5·29. Hence the mixture has become slightly alkaline during the process. Therefore there can be no question of the production of any considerable amount of acid. On the other hand, a preliminary qualitative test showed that we have to deal with the production of carbon dioxide. We also found that the solutions obtained in the decomposition of the nicotine had a remarkably strong odour of pyridine and methylamine. With KI—HgI_2 reagent we obtained the familiar white precipitate which is given by solutions of pyridine and which is soluble in excess of the precipitating solution (see previous paper). In all probability the nicotine here undergoes a decomposition process with the formation of pyridine, methylamine and carbon dioxide as end products.

Hence the process must be interpreted as the effect of an enzyme, of the class of oxidases, on the nicotine.

In how far this is to be ascribed to the action of a specific oxidase will be shown by later experiments.

Since the solutions, kept in the thermostat for 2 hours at 37°, showed no change in appearance under the conditions we employed, there can be no possibility of bacterial action.

Experimental.

In the quantitative determination of the decomposition products of nicotine, produced by the enzyme action of macerates and expressed juices of dried and fermented tobacco leaves, the volatile non-nicotine nitrogen which is produced under their influence during 2 hours at 37° at neutral reaction was estimated by the method described in the previous article. The materials necessary for this were of the following description.

As substrate we made use of a solution of nicotine (Nikotin, pur, Merck) in water, of which the titre towards sodium alizarinsulphonate as indicator was determined. For the calculation, the figure thus obtained has to be multiplied by two, since only one nitrogen atom can be titrated, whilst in the decomposition of the nicotine, both N atoms of the nicotine molecule become converted into volatile non-nicotine nitrogen.

The enzyme extracts were prepared as follows:

(*a*) Expressing the juice from freshly gathered and finely chopped tobacco leaves (obtained from the Agricultural School at Mikveh Israel, near Jaffa)[1] and diluting it ten times.

[1] For which we have to thank the Director, Mr Krause.

(*b*) Preparing extracts from finely powdered dried green tobacco leaves of the same variety as in (*a*); the drying took place in a vacuum drying oven at about 30°.

(*c*) Making macerates from slowly dried powdered tobacco leaves, of a brown colour, as they were supplied from the stores of Messrs Lubliner at Rosh-Pinah in Upper Galilee[1].

(*d*) Preparing macerates from finely cut cured tobacco as used by Messrs Maspero in their cigarette manufactory.

The macerates were prepared by allowing 10 g. of the tobacco together with ten times the quantity of water to stand at room temperature for 6 hours and then filtering.

I. Experiments with the Expressed Juices.

1. *Peptolytic ferment.*

In order to detect effects due to peptolytic ferments 5 cc. of the juice after neutralisation were treated with 10 cc. peptone and 5 cc. disodium hydrogen phosphate as buffer.

The amino N titration was carried out by Sörensen's method and the following values were obtained:

$$t_0 = 13{\cdot}85, \qquad t_2 = 14{\cdot}7, \qquad t_4 = 14{\cdot}8.$$

The control test showed no change.

It is thus evident that a progressive, although slow, hydrolysis of the peptone occurs.

2. *Nicotine-splitting ferment.*

(*a*) The following solution was used:

5 cc. nicotine (= 6·1 cc. $N/10$ H_2SO_4) + 5 cc. expressed juice + 6·1 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine showed a titre of 26 cc. $N/10$ H_2SO_4 corresponding to the content of volatile non-nicotine nitrogen.

(*b*) In this case the following mixture was used:

5 cc. nicotine (= 16·2 cc. $N/10$ H_2SO_4) + 20 cc. expressed juice (made neutral to litmus) + 16·2 cc. $N/10$ H_2SO_4 (for neutralisation).

The solution was made up to 100 cc. and 20 cc. of it taken for titration.

Calculated quantity of nicotine = 3·24 cc. $N/10$ H_2SO_4. Non-nicotine N in the filtrate from the nicotine precipitate = 2·6 cc. $N/10$ H_2SO_4.

(*c*) 5 cc. expressed juice contain a quantity of

nicotine + non-nicotine N corresponding to 0·28 cc. $N/10$ H_2SO_4.

Hence, even if the total alkalinity of the juice should be considered as corresponding to non-nicotine nitrogen, there are still in the distillates, after precipitating the nicotine, non-nicotine bases originating from the nicotine corresponding to (2·6 − 0·28) cc. = 2·32 cc. $N/10$ H_2SO_4.

[1] We have to acknowledge our obligations to the Directors of the firm of Lubliner, and especially Dr Zlocisti, for their valuable assistance.

The extent to which the process goes on may be calculated as follows:

We commenced with a quantity of nicotine corresponding to titre value of 3·24 cc. $N/10$ H_2SO_4. This means a total N content corresponding to 6·48 cc. $N/10$ H_2SO_4.

From this there resulted nitrogen products, in the form of volatile bases, corresponding to a titre of 2·32 cc. $N/10$ H_2SO_4.

The process therefore occurs to the extent of 35 %.

II. Experiments with the Macerates.

3. *Nicotine-splitting ferment in macerates from dried, brown, tobacco.*

The following solution was used:

5 cc. nicotine (=15 cc. $N/10$ HCl) + 5 cc. macerate + 15 cc. $N/10$ H_2SO_4 (for neutralisation).

The mixture was made up to 50 cc. and 20 cc. of this taken for the experiment.

Calculated quantity of nicotine	=6 cc. $N/10$ H_2SO_4
Non-nicotine N in the distillate after precipitating the nicotine	=1·9 cc. $N/10$ H_2SO_4

2 cc. of the maceration liquid contain:

(Nicotine + non-nicotine) N	=0·53 cc. $N/10$ H_2SO_4
∴ Volatile non-nicotine N arising from nicotine	=1·37 cc. $N/10$ H_2SO_4

From a solution of nicotine corresponding to a titre of 6 cc. $N/10$ H_2SO_4 and therefore containing total N equivalent to 12 cc. $N/10$ H_2SO_4, there was thus produced a quantity of non-nicotine bases equivalent to 1·37 cc. $N/10$ H_2SO_4; hence decomposition must have occurred to the extent of 11·4 %.

4. *Comparative investigation of the nicotine-splitting ferments in the macerates obtained from unfermented, dry, green and brown tobacco, and from dried, cured tobacco.*

(*a*) *Dried green tobacco leaves* (unfermented).

10 g. of dry green tobacco leaf were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 1·5 cc. $N/10$ H_2SO_4; non-nicotine N = 1·3 cc. $N/10$ H_2SO_4.

For the purpose of the test a solution was made up containing:

10 cc. macerate as above + 5 cc. nicotine (=3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine contained non-nicotine N corresponding to 1·9 cc. $N/10$ H_2SO_4.

The balance-sheet of the decomposition of the nicotine is therefore as follows:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	=0·75
Nicotine in the substrate	=3·00
Calculated total nicotine	=3·75
Non-nicotine N in the macerate	=0·65
Non-nicotine N in the substrate + macerate	=1·9
Non-nicotine N of substrate, by difference	=1·25

Since from a solution of nicotine, equivalent to 3·75 cc. $N/10$ H_2SO_4, which therefore actually contains nicotine N equivalent to 7·5 cc. $N/10$ H_2SO_4, there was produced non-nicotine N corresponding to 1·25 cc. $N/10$ H_2SO_4, the process has gone on to the extent of 16·7 %.

(*b*) *Dried brown tobacco leaf* (unfermented).

10 g. tobacco were treated with 100 cc. water and then filtered.

20 cc. of macerate contained:

Nicotine N = 1·2 cc. $N/10$ H_2SO_4; non-nicotine N = 1·8 cc. $N/10$ H_2SO_4.

For the test the following solution was used:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4) + 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate, after precipitation of the nicotine, contained non-nicotine N = 3·0 cc. $N/10$ H_2SO_4.

Thus we have:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	= 0·6
Nicotine in the substrate	= 3·0
Total nicotine	= 3·6
Non-nicotine N in macerate + substrate	= 3·0
Non-nicotine N in the macerate ...	= 0·9
Non-nicotine N in the substrate ...	= 2·1

Thus, in a solution containing nicotine showing a titre for N of 3·6 cc. $N/10$ H_2SO_4 and therefore actually corresponding to a total N content equivalent to 7·2 cc. there was produced non-nicotine N corresponding to 2·1 cc. $N/10$ H_2SO_4; hence the process has gone on to the extent of 29 %.

(*c*) *Fermented tobacco.*

10 g. of this tobacco were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 2·2 cc. $N/10$ H_2SO_4; non-nicotine N = 2·6 cc. $N/10$ H_2SO_4.

The following solution was made up for the test:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4) + 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine was found to contain non-nicotine N corresponding to 2·0 cc. $N/10$ H_2SO_4.

The data for the nicotine splitting are:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	= 1·1
Nicotine in the substrate	= 3·0
Total nicotine	= 4·1
Non-nicotine N in macerate + substrate	= 2·0
Non-nicotine N in macerate	= 1·3
Non-nicotine N in the substrate ...	= 0·7

Since a solution containing nicotine giving a titre for N of 4·1 cc. $N/10$ H_2SO_4 and therefore actually containing total N equivalent to 8·2 cc. gave rise to non-nicotine N corresponding to 0·7 cc. $N/10$ H_2SO_4, the process has gone on to the extent of 8½ %.

Summary and Discussion of Results.

From the experiments described in II. 4. (*a*)–(*c*), which were carried out under similar conditions, it appears that the fermentative effect is greater in the case of unfermented tobacco leaves allowed to dry naturally than in the case of tobacco obtained by drying rapidly. Of course the comparison is not absolute, since the latter may still contain not inconsiderable amounts of water. The effectiveness of an equal amount of fermented tobacco in comparison is remarkably slight.

Thus it seems that the greater part of the activity disappears during the fermentation process. These phenomena remind us very strongly of the results which in the meantime have been observed in this Institute in connection with the alteration in polypeptide-splitting activity during the germination process.

Here also the stability of the enzyme system becomes much reduced.

It will be our object in further investigations to study more definitely the identity of the products of decomposition of the nicotine, as also the rôle of the oxidase effect occurring during the process of fermentation.

We have to acknowledge our thanks to Miss M. Goldberg and Miss S. Beham for their assistance in carrying out the determinations entailed in this work.

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CXXV. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

II. CARBOHYDRATE METABOLISM.

By ERIC GORDON HOLMES (*Grocers' Company Research Scholar*) and BARBARA ELIZABETH HOLMES.

From the Biochemical Laboratory, Cambridge.

(*Received August 28th, 1925.*)

In a previous communication [B. E. and E. G. Holmes, 1925] we were able to show that the "resting" lactic acid content of rabbits' brains was greatly reduced, if the animals had convulsed, or were on the point of convulsing, as a result of a dose of insulin.

That the low values obtained as a result of insulin administration might possibly have been due to an increased oxidation of lactic acid as a consequence of the presence of the insulin, is suggested by the work of Neuberg, Gottschalk and Strauss [1923] who demonstrated an increased formation of acetaldehyde (which might have come from the oxidation of lactic acid) in tissue hashes to which insulin had been added. We found, however, reason to suppose that, although reducing substance can be extracted from rabbits' brains after death, none of it (or at most a very small amount) is glucose. Whilst we found that intact or chopped brain does not by itself give rise to increased lactic acid production on standing at body p_H, we confirmed the statement of Warburg, Posener and Negelein [1924] that it does so if glucose be added to it. The suggestion therefore presented itself that the "resting" lactic acid values obtained for brain were ultimately dependent on the level of the blood-sugar, and not due to a direct effect of insulin, or any accompanying impurity, on the brain cells.

Since our first experiments, we have found it possible to obtain, by suitable means, values for brain lactic acid very considerably higher than any "normal" values, and this without any marked rise of blood-sugar. In spite of this, however, we are inclined to believe that the lowering of the brain lactic acid after insulin is a consequence of the fall in blood-sugar, and does not occur apart from the latter.

It was at first hoped that it would be possible to plot a brain lactic acid—blood-sugar curve. For reasons to be discussed immediately, this could only be done, if at all, by using an unjustifiably large number of animals. It is, however, easy to show that the brain lactic acid does not fall appreciably

until the blood-sugar has dropped considerably, whilst, provided that the blood-sugar has fallen below about 70 mg. per 100 cc., the resting lactic acid value is always well below the normal.

The experimental results are arranged in two tables. Table I shows the results obtained from groups of animals from the same litter. (The normal values from animals of the same litter show much less variation than those from animals taken at random.) Table II contains values obtained from animals used for experiments performed with another object, in which the brain lactic acid and the blood-sugar were both determined.

No. 5 β deserves particular attention. The animal was not starved, and was therefore given a large dose of insulin (30 units). Convulsions took place after 98 minutes. After these had lasted 5 minutes 8 cc. of 10 % glucose in normal saline were given subcutaneously. The animal recovered. Twenty minutes later, blood samples were taken, and the animal was killed. The blood-sugar was 119 mg. per 100 cc., while the brain lactic acid was 56 mg. per 100 g. This last value is higher than any obtained for convulsed animals, and is evidently rising towards the normal.

Table I.

Litter no.	Remarks		Dose of insulin (units)	Interval before killing (minutes)	Blood-sugar mg. per 100 cc.	Brain lactic acid ("Resting") mg. per 100 g
Y 1	Normal, starved		—	—	138	95
Y 2	Insulin, starved		20	40	107	88
Y 5	,, ,,		10	—	92	93
Y 3	,, ,,		10	80	65	49
Y 6	,, ,,	C	10	—	68	40
Y 4	,, ,,	C	10	100	—	44
2 α	Insulin, starved		10	63	95	76
4 α	,, ,,	C	10	82	—	29
1 α	,, fed	C	40	422	50	45

"C" = convulsed.

Table II.

No.	Remarks	Blood-sugar mg. per 100 cc.	Resting brain lactic acid mg. per 100 g.
X 1	Insulin	124	96
13 F	Normal	150	101
17 D	,,	131	75
23	Insulin	61	44
5 β	Insulin, convulsed. Recovered with 8 cc. 10 % glucose	119	56

The blood was drawn from an ear vein.
Blood-sugar by the method of Hagedorn and Jensen [1923].
Brain frozen and crushed before weighing [B. E. and E. G. Holmes, 1925].
Lactic acid by method of Meyerhof [1920]. (Uncorrected figures.)

Since it is seldom possible to obtain a litter of more than six rabbits, any single series of values is limited to that number. This makes it extremely difficult to hit off any required blood-sugar level, for the animal must be

killed immediately the sample has been taken, and long before the sugar estimation can be completed.

It will be observed that the lactic acid does not fall until the blood-sugar is below about 70 mg. per 100 cc. Since values ranging from 100–75 mg. per 100 g. (we have had two values as low as 65) are obtained for the resting brain lactic acid of normal animals from different litters, it is hardly to be expected than an exact correlation between blood-sugar and brain lactic acid could be found above this level.

The fact that, for instance, rabbit "Y 2" (Table I) showed no appreciable fall in brain lactic acid 40 minutes after 10 units of insulin, shows that insulin, of itself, exerts no direct effect on the brain lactic acid metabolism.

It is, of course, certain that there are many factors complicating the issue. If the amount of lactic acid formed depends upon the amount of glucose made available *via* the blood stream, it must vary, not only with the level of the blood-sugar, but also with the rate of blood supply to the brain, and, further, by the rate at which glucose is able to diffuse into the cell, and reach the point at which chemical change occurs. Of these three factors, one only is known, namely, the blood-sugar level.

Apart from these considerations, Hiller and collaborators have recently shown [Hiller, Almer, Linder and Van Slyke, 1925] that only a part of the reducing substance, estimated in blood by the methods in general use, is glucose. A variable but not inconsiderable part of it appears to be some material or materials which is not fermented by yeast, nor destroyed by the glycolytic enzyme of the blood itself. In fact, they suggested that after insulin the glucose content of rabbits' blood may fall to zero, though a very definite reducing power still remains. If these findings be correct, it is hardly likely that the figures appearing in our tables under "blood-sugar" represent at all accurately the glucose content of the blood.

We feel, however, that the experiments here described make it improbable that the fall in brain lactic acid which we observe is due to an immediate effect of the insulin, or of any substance injected along with it, on the brain cells. In the latter connection, it is worth mentioning that in our earlier experiments we used a dried insulin preparation (supplied by Messrs Boots); in those here described, we employed a liquid preparation prepared by Burroughs and Wellcome for clinical use.

Summary.

A comparison of the blood-sugar values, and the resting lactic acid content of the brains of rabbits, shows that the fall in the latter resulting from the injection of insulin does not occur until the blood-sugar has reached a fairly low level. At the convulsive or flaccid (pre-convulsive) stage the brain lactic acid content is very small. One experiment indicates that the lactic acid content rises again when the animal has recovered as the result of injection of glucose.

From these results it appears probable that the fall in the resting lactic acid content of the brain after insulin injection is not due to a direct effect of insulin in promoting increased oxidation of lactic acid, nor to any direct effect of insulin or an accompanying impurity in depressing the production of lactic acid by the brain cells, but is rather caused by the fall in the blood-sugar level, and the resulting shortage of glucose in the brain.

One of us (B. E. H.) is in receipt of a grant from the Medical Research Council.

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CXXVI. THE ESTIMATION OF FAT IN BLOOD.

By CORBET PAGE STEWART (*Beit Memorial Research Fellow*) and ADAM CAIRNS WHITE.

From the Departments of Therapeutics and Pharmacology, University of Edinburgh.

(*Received August 31st, 1925.*)

In view of the large number of pathological conditions in which the fat content of the blood is increased, and of our relatively meagre knowledge of fat transport and metabolism, it is eminently desirable than an accurate, and, as far as possible, non-subjective method be devised for the estimation of fatty acids in blood. The methods at present available fall into two classes: first, nephelometric, of which that of Bloor [1914] is typical; second, oxidative, of which those of Gade [1919] and Bang [1918] are typical.

Nephelometric methods are open to several objections. The personal factor is comparatively great, and the time factor is important since the suspension of fat flocculates at a measurable and varying rate [Blix, 1924]. Moreover, Bloor's method estimates both cholesterol and fatty acids, the latter being ultimately determined by difference. This last objection, of course, does not extend to the more recent method of Bloor, Pelkan and Allen [1922].

In Gade's method [1919] the fat extracted from the dried blood by ether is allowed to react for a definite time with an excess of potassium permanganate, the excess being then determined by the addition of excess of oxalic acid and back-titration with permanganate. The method is somewhat cumbersome, the reaction with permanganate must be carefully controlled with regard both to time and temperature, and an empirically determined correction has to be applied. Bang's method [1918], in which the fat is oxidised by chromic acid, the excess being determined by allowing it to act on potassium iodide and titrating the liberated iodine with thiosulphate, gave good results in the hands of Gade, as reported in his comprehensive review [1919]. As in the case of Bloor's method, however, it gives both cholesterol and fat, and the true fat content is obtained by estimating the cholesterol separately and subtracting.

Hitherto, the small amount of fatty acid obtainable from the 2 cc. of blood usually available for routine estimations has rendered it impossible to utilise, with any degree of accuracy, the hydrolytic method of estimation in which the fat is saponified by sodium hydroxide and the excess alkali titrated with acid. The new micro-burette described by Rehberg [1925], however, delivers

0·10 cc. of solution and can be used for titrations requiring fractions of that amount with at least as great accuracy as is attainable with the 10 cc. of the macro-titration. Since 0·10 cc. of $N/10$ NaOH is equivalent to 26·86 mg. of tripalmitin or 29·67 mg. of tristearin, the burette can evidently be used to titrate the fatty acids liberated by hydrolysis from the fat contained in only 1·0 cc. of normal blood.

Acting on these considerations, we attempted to estimate known amounts of synthetic tripalmitin. This was prepared from pure palmitic acid and glycerol by the method of Scheij [1899], purified by repeated extraction with alcohol, and many recrystallisations from alcohol-ether mixture, and finally from pure ether. Quantities were taken corresponding to the amounts present in 1·0 cc. of normal and lipaemic bloods (3–18 mg.). For this purpose the fat was dissolved in pure anhydrous ether and the requisite quantities of the solution were measured into 50 cc. round-bottomed flasks. After evaporation of the ether, 5 cc. of $N/10$ NaOH and 5 cc. of absolute alcohol were added, and the mixture allowed to evaporate almost to dryness on the water-bath, a process which occupied about 2 hours. We found it advisable at this stage not to attempt the direct titration of the excess of sodium hydroxide, for during the manipulation before and after hydrolysis a small amount of carbon dioxide is inevitably absorbed. Since the volumes involved in the titration are so small, and an indicator such as phenolphthalein must be used, this introduces a serious error. After hydrolysis we therefore added to the mixture 5 cc. of $N/10$ HCl. This exactly neutralised the whole of the sodium hydroxide, and liberated the fatty acids which had been produced during the hydrolysis. We then boiled down to about 1 cc. in order to drive off the absorbed carbon dioxide. The residual liquid was transferred quantitatively to a 10 cc. volumetric flask, absolute alcohol being used for the washings and for making up the volume to 10 cc. 1 cc. of this final solution, corresponding to one-tenth of the amount of fat originally taken, was titrated with $N/10$ sodium hydroxide from the Rehberg burette. The sodium hydroxide used in the titration must, of course, be free from carbonate. In our experiments, it was kept in a paraffined bottle provided with a syphon and tap, the air inlet being guarded by a soda-lime tower. In filling the burette, a short rubber tube connected the burette nozzle and the syphon. In this way the standard NaOH was prevented from coming into contact with the air. The indicator used was phenolphthalein, and a correction was applied for the amount of indicator, volume of fluid, etc. as described by Rehberg [1925].

It cannot be over-emphasised that for these estimations both reagents and apparatus must be accurately standardised. Thus, if the hydrochloric acid be slightly below $N/10$, or if slightly less than 5·0 cc. be added, the fatty acids will be incompletely liberated. Conversely, if acid be added in slight excess, it will remain to be neutralised in the titration. In either case, apparently slight variations may lead to serious errors, notwithstanding the subsequent dilution, since the volume of the titrating fluid is so small. Careful

manipulation is necessary at every stage, but granted this, as our results abundantly show (Table I), not only do duplicate titrations from the same sample agree, but duplicate samples, treated separately throughout, give results within 5 % of the theoretical. We may point out that in several of the duplicate results recorded, one of the estimations has been carried out by each of us. An important advantage of the method is that in case of necessity, as many as eight or nine titrations can be made on each sample.

Table I. *Estimation of fat in pure solutions of tripalmitin.*

Fat present g. per 100 cc.	Titre (corrected)*	Fat found g. per 100 cc.	Error %
0·300	10·3		
	10·8	0·283	5·6
	12·0		
	11·3	0·310	3·3
0·600	20·5		
	20·4	0·596	0·6
	22·8	0·618	3·0
	23·2		
0·800	29·8		
	29·8	0·800	0·0
0·900	34·0		
	34·6	0·921	2·3
1·200	43·8		
	43·6	1·174	2·2
	43·8		
	44·4	1·185	1·2
1·500	53·9		
	53·7	1·445	3·6
1·800	68·9		
	70·0	1·765	2·0
	69·6		
	69·8	1·770	1·7

* The correction derived from macro- and micro-titration of the acid and alkali used, with a standard amount of titration fluid (1 cc.) and a standard amount of indicator, was 0·7.

In extending the method to blood, we have not concerned ourselves with the selective extraction by different solvents of the various constituents of the blood-fat, but have contented ourselves with showing its applicability to the estimation of the total fatty acids of the blood irrespective of the state of combination in which they occur. 2·0 cc. of blood were added with constant shaking to about 30 cc. of alcohol-ether mixture (three parts alcohol, one part ether) in a 50 cc. flask. The mixture was brought just to boiling point, cooled, and made up to 50 cc. with alcohol-ether. After thorough mixing, it was allowed to settle and 25 cc. of the clear supernatant liquid were withdrawn for analysis. This procedure was adopted to avoid the concentration of the extract which may take place, to a varying degree, during filtration. The remainder of the procedure was exactly as described for pure solutions of fat. Table II gives some of the results we have obtained in estimating fat in blood, alone, and with added fat.

Notwithstanding the agreement of duplicate analyses, there still remains the possibility that the extract may contain, in addition to fat and fatty acids, some acidic substances soluble in aqueous alcohol-ether mixtures as well as

in water. In this category would be included such substances as acetoacetic acid, hydroxybutyric acid, etc. These, though present only in traces in normal blood, may occur to an appreciable extent in pathological specimens. They are, however, of low boiling point, and are, moreover, volatile in steam, so that they are removed by the boiling down after the addition of the hydrochloric acid. To ensure that interfering substances are absent, or are present in negligible quantities if at all, we carried out the following experiment. 50 cc. of the alcohol-ether extract from blood sample No. I (Table II), which was a pathological specimen, were evaporated to dryness on the water-bath. The residue was extracted with two 1 cc. portions of distilled water. The aqueous extract, after filtration, was boiled, and titrated with $N/10$ NaOH from the Rehberg burette. The extract was acid to phenolphthalein, indicating the absence of basic interfering substances, but was rendered distinctly alkaline by the addition of 0·004 cc. of $N/10$ NaOH. We may therefore conclude that the alcohol-ether extract contained no interfering acidic substances.

Table II.

No. of blood sample	Fat added g./100 cc.	Found g./100 cc.	Fat content of blood g./100 cc.	Mean	Error %
I. Ox blood	0·900	2·083	1·183	—	1·5
	1·500	2·698	1·198	—	—
	1·500	2·703	1·203	—	0·5
	1·500	2·706	1·206	1·198	0·7
II. Mixed specimen of human blood —pathological	—	1·845	1·845	—	0·5
	—	1·821	1·821	—	2·0
	—	1·872	1·872	—	1·0
	—	1·877	1·877	1·854	1·0
	0·300	2·130	1·830	—	1·5
	0·300	2·122	1·822	—	2·0
	1·200	3·073	1·873	—	1·0
	1·500	3·427	1·927	—	4·0
III. Human blood— normal	—	0·306	0·306	0·312	1·8
	—	0·317	0·317	—	1·8

In this table the fat has been calculated as tripalmitin.

We have attempted to modify the method so as to permit of using much smaller quantities of blood. Even with careful technique, however, the errors in measuring with the ordinary pipette are sufficient to vitiate the results. By substituting for pipettes measuring instruments based on the principle of the Rehberg burette, it would, no doubt, be possible to obtain moderately accurate results with quantities of blood less than 0·5 cc. The method, even as we have used it, calls for very careful work, and can hardly be used as a routine method by the busy technician; it is primarily a method for research. However, the use of a modified Rehberg burette for the measurement of the alkali used in hydrolysis and the acid subsequently added renders the method less delicate and more suitable for routine use.

The expenses of this work have been partly defrayed by a grant from the Moray Fund of this University, to the Trustees of which our thanks are due.

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CXXVII. A SYNTHESIS OF *N*-METHYL-PUTRESCINE AND OF PUTRESCINE.

By HAROLD WARD DUDLEY
and WILLIAM VEALE THORPE.

From the National Institute for Medical Research, Mount Vernon, Hampstead, N.W. 3.

(*Received September 1st, 1925.*)

In connection with other work proceeding in this laboratory it became necessary to prepare the hitherto unknown *N*-methylputrescine, and it was hoped that this compound would result from the action of methylamine on δ-bromobutylamine thus; $Br(CH_2)_4NH_2 \xrightarrow{CH_3NH_2} CH_3.NH.(CH_2)_4.NH_2$. Unfortunately, under all experimental conditions tried, the effect of methylamine on this and the corresponding iodo-compound was similar to that of sodium hydroxide [von Braun and Beschke, 1906; Gabriel, 1909], ring closure occurring with exclusive formation of pyrrolidine.

It was found, however, that δ-iodo- (and δ-bromo-) butylamine can be benzoylated almost quantitatively and that the monobenzoyl-δ-iodobutylamine obtained can then be converted by the action of methylamine into monobenzoyl-*N*-methylputrescine, from which the base itself results on hydrolysis with hydrochloric acid.

$NH_2.(CH_2)_4.I \longrightarrow C_6H_5.CO.NH.(CH_2)_4.I \longrightarrow C_6H_5.CO.NH.(CH_2)_4.NH.CH_3 \longrightarrow NH_2.(CH_2)_4.NH.CH_3$.

By the use of ammonia in place of methylamine, putrescine itself can be prepared, and this synthesis may prove useful to others, as it did to us, on account of the difficulty experienced in obtaining pyrrole which is the starting point of the usual synthesis due to Ciamician and Zanetti [1889, 1890] and Willstätter and Heubner [1907]. The synthesis described in this paper has the further advantage that it involves the preparation of the hitherto undescribed *mono*benzoylputrescine which is inaccessible from other directions and may be useful in further synthetic work.

The secondary base di-δ-benzoylaminobutylamine

$$NH[(CH_2)_4.NH.CO.C_6H_5]_2$$

is also formed during the interaction of ammonia and benzoyl-δ-iodobutylamine; the base di-δ-aminobutylamine has been prepared from this by hydrolysis and is described in the experimental section.

N-Methylputrescine has also been synthesised in the following way:

$$\mathrm{Br.(CH_2)_3.OC_6H_5} \xrightarrow{\mathrm{CH_3NH_2}} \mathrm{CH_3.NH.(CH_2)_3.OC_6H_5} \xrightarrow{\mathrm{HBr}}$$

$$\mathrm{CH_3.NH.(CH_2)_3.Br.} \xrightarrow{\mathrm{C_6H_5.COCl}} \mathrm{CH_3.N.CO.C_6H_5.(CH_2)_3Br} \xrightarrow{\mathrm{KCN}}$$

$$\mathrm{CH_3.N.CO.C_6H_5.(CH_2)_3.CN} \xrightarrow{\mathrm{Na+C_2H_5OH}} \mathrm{CH_3.NH.(CH_2)_4.NH_2}.$$

Difficulties were, however, encountered in the preparation of the nitrile, and the best experimental conditions for the synthesis were not studied in detail.

Experimental.

δ-Bromo- and δ-iodobutylamine were prepared from α-γ-dibromopropane according to the directions of von Braun and Beschke [1906] and Gabriel [1909].

The hydrochloride and picrate of phenoxybutylamine, one of the intermediate substances in the preparation, melt at 157·5° (corr.) and 159·5° (corr.) respectively.

Benzoyl-δ-bromo- and benzoyl-δ-iodobutylamine. δ-Bromo- and δ-iodobutylamine are benzoylated by adding 2 mols. benzoyl chloride to an aqueous solution of the amine halide cooled in ice. Solid sodium bicarbonate is then gradually added with constant shaking, the liquid being kept at 0°. The solid benzoyl derivative is extracted with ether, washed with dilute sulphuric acid and dilute potash and dried over potassium carbonate. Evaporation of the ether leaves colourless needles. Benzoyl-δ-bromobutylamine recrystallised from ether melts at 52° (corr.). Benzoyl-δ-iodobutylamine agrees in its properties with that prepared by von Braun and Beschke [1906] from benzoylpyrrolidine. Yield 90 %.

Monobenzoyl-N-methylputrescine. 13 g. benzoyl-δ-iodobutylamine are dissolved in 60 cc. absolute alcohol and boiled gently under a reflux condenser with 30 cc. 33 % alcoholic methylamine for 2 hours. The alcohol and methylamine are removed *in vacuo* on the water-bath. The resulting syrup is dissolved in water and the base liberated with NaOH. The insoluble oil is extracted with chloroform. Evaporation of the dried extract leaves a pale oil insoluble in water and sparingly soluble in ether, but readily soluble in alcohol. The addition of HCl gives a syrupy hydrochloride which can be precipitated from alcoholic solution by ether in colourless needles melting at 150°. On benzoylation in the ordinary way the base gives dibenzoyl-*N*-methylputrescine.

N-Methylputrescine dihydrochloride. The syrupy monobenzoyl-*N*-methylputrescine is hydrolysed by heating it at 130° for 8 hours in a sealed tube with five times its weight of concentrated HCl. The hydrochloride may not crystallise at once but comes out on standing with absolute alcohol for a day. Yield 70 %, calculated from benzoyliodobutylamine. The hydrochloride is very

soluble in water but insoluble in alcohol and acetone. Recrystallised from 95 % alcohol it forms hygroscopic needles melting at 179° (corr.).

Analysis.

	Found %	Calculated for $C_5H_{14}N_2.2HCl$ %
C	34·11	34·29
H	8·86	9·14
N	15·99	16·00
Cl	40·52	40·57

N-*Methylputrescine*, liberated from aqueous solution of the hydrochloride by means of alkali, can be extracted with chloroform and is a colourless oil boiling at 161–163°, very soluble in water and almost insoluble in ether. It has a strong smell, similar to piperidine but not so pungent and fumes in moist air. The *dipicrate*, recrystallised from water, forms prisms or needles melting with decomposition at 229–230·5° (corr.). It is almost insoluble in alcohol and cold water.

Analysis. Picric acid: found (by nitron) 81·78 %; calc. for

$$C_5H_{14}N_2.2C_6H_3O_7N_3 \ 81{\cdot}79\ \%.$$

The *chloroaurate*, recrystallised from *N*/10 HCl, forms deep yellow prisms, containing 1 mol. water of crystallisation, melting at 192° (corr.) with previous softening, and decomposing at 215° (corr.).

Analysis. Au: found 49·24 %; calc. for $C_5H_{14}N_2.2HAuCl_4.H_2O$ 49·27 %. After drying between 100 and 110°, Au: found 50·53 %; calc. for

$$C_5H_{14}N_2.2HAuCl_4 \ 50{\cdot}41\ \%.$$

H_2O: found 2·55 %; calc. for $1H_2O$ 2·25 %. (Slight loss of HCl.)

The *chloroplatinate*, recrystallised from dilute HCl, forms reddish prisms decomposing at 230·5° (corr.). It can be precipitated from aqueous solution as a crystalline powder by alcohol.

Analysis. Pt: found 38·01 %; calc. for $C_5H_{14}N_2.H_2PtCl_6$ 38·10 %.

The *picrolonate*, recrystallised from water, forms needles decomposing between 254° and 265° according to the rate of heating. It is very slightly soluble in alcohol.

The *mercuric chloride compound* is precipitated from alcoholic solution in needles changing to plates. Recrystallised from water it melts at 148° (corr.). It is sparingly soluble in alcohol.

The *dibenzoyl compound* is very soluble in chloroform and alcohol. It is precipitated from chloroform by ether in small needles, and larger needles are obtained by slow crystallisation from dilute alcohol. M.P. 115·5° (corr.).

Monobenzoylputrescine. 10 g. benzoyl-δ-iodobutylamine are heated in a sealed tube with 6 g. ammonia in 50 cc. alcohol for 4 hours at 100°. The alcohol and excess ammonia are removed *in vacuo* on the water-bath. The resulting oil is stirred up with warm dilute HCl and shaken with ether. The aqueous layer is contaminated with an ether-insoluble oil which must be entirely removed by repeated filtration. The colourless solution thus obtained is made

alkaline with NaOH and extracted with chloroform. Evaporation of the dry extract gives a pale oil which rapidly absorbs CO_2. On dissolving this oil in the requisite amount of 10 % HCl to give a neutral solution, a hydrochloride gradually comes down as a white crystalline powder. The deposition of this salt is accelerated by immersing the solution in a freezing mixture. This is di-δ-benzoylaminobutylamine hydrochloride and is removed by filtration when separation is complete. The filtrate can be treated with benzoyl chloride and NaOH in the usual manner when dibenzoylputrescine immediately separates as a white solid which can be filtered off and hydrolysed, giving putrescine hydrochloride. Yield of dibenzoylputrescine 50 %. For the isolation of *monobenzoylputrescine hydrochloride* the filtrate from the di-δ-benzoylaminobutylamine hydrochloride is concentrated and poured into 800 cc. acetone when monobenzoylputrescine hydrochloride separates in needles. Yield about 40 %. The remainder can be isolated by repeating the acetone treatment several times. Monobenzoylputrescine hydrochloride is soluble in water and alcohol. Recrystallised from alcohol, it melts at 171° (corr.).

Analysis. Cl: found 15·40 %; calc. for $C_{11}H_{17}ON_2Cl$ 15·51 %.

The base is an oil slightly soluble in water, insoluble in ether but very soluble in chloroform and alcohol.

The *picrate* forms prisms sparingly soluble in water and alcohol. Recrystallised from water it melts at 173° (corr.).

Putrescine dihydrochloride. Monobenzoyl- (or dibenzoyl-) putrescine is hydrolysed by heating in a sealed tube for 8 hours at 130° with five times its weight of concentrated HCl. The product is dissolved in boiling water and cooled. Benzoic acid is filtered off and the filtrate extracted with ether to remove the remainder. The aqueous layer is evaporated on the water-bath, leaving a white crystalline mass. This is washed on to a filter with a little absolute alcohol. Yield of putrescine hydrochloride (M.P. 300°) from crude dibenzoyl compound 73 %. A further 11 % can be recovered from the filtrate by evaporating and re-treating with alcohol. The yield from pure monobenzoylputrescine hydrochloride is theoretical.

From the hydrochloride the picrate, chloroaurate and chloroplatinate were prepared. The melting points agreed with those given in the literature.

Analyses. Chloroaurate. The salt lost 2 mols. H_2O on drying at 95°. Au: found 51·29 %; calc. for $C_4H_{12}N_2.2HAuCl_4$ 51·34 %. *Chloroplatinate.* Pt: found 38·99 %; calc. for $C_4H_{12}N_2.H_2PtCl_6$ 39·19 %.

Di-δ-benzoylaminobutylamine hydrochloride, recrystallised from alcohol, forms prisms, M.P. 233° (corr.). When mixed with monobenzoylputrescine hydrochloride the melting point is depressed to 171°, the melting point of pure monobenzoylputrescine hydrochloride. It is sparingly soluble in cold water and alcohol. Yield 10 %.

Analysis. Cl: found 8·81 %; calc. for $C_{22}H_{30}O_2N_3Cl$ 8·78 %.

The *base* is precipitated from a solution of the hydrochloride by addition of sodium hydroxide solution in needles melting at 68°. It is very soluble in

alcohol and chloroform but sparingly in water and ether. It is purified by precipitation from alcohol by water.

The *picrate*, recrystallised from very dilute acetone, forms needles melting at 121° (corr.). It is sparingly soluble in water and alcohol.

Di-δ-aminobutylamine hydrochloride is obtained by hydrolysis of the dibenzoyl compound with HCl under the conditions employed for the hydrolysis of monobenzoylputrescine hydrochloride. It is very soluble in water but insoluble in alcohol. Recrystallised from dilute alcohol it forms needles softening indefinitely at about 290° with blackening.

The *base* is an oil smelling like putrescine and fuming in moist air. It is very soluble in water and chloroform. It is not attacked by boiling 25 % NaOH and is not volatile in steam.

The *tripicrate* forms long thin plates, often resembling needles, melting with decomposition at 255° (corr.) after recrystallisation from water. It is sparingly soluble in alcohol.

Analysis. Picric acid: found (by precipitation with nitron) 81·37 %; calc. for $C_8H_{21}N_3.3C_6H_3O_7N_3$ 81·19 %.

The *chloroaurate*, recrystallised from *N*/10 HCl, forms prisms melting at 209° (corr.) with decomposition.

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CXXVIII. A NEW HYDROLYSIS PRODUCT FROM ELASTIN.

By RUDOLF ENGELAND.

Munich.

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It is generally believed that Millon's reaction obtained with proteins is due to the presence of tyrosine. However, such a substance as gelatin also gives this reaction, although no tyrosine is formed upon hydrolysis. Similar conditions prevail in the case of elastin, which in its chemical composition shows some relation to gelatin; no tyrosine could be isolated from the crystallised crude leucine originally obtained from elastin in a joint investigation by W. Biehler and myself[1]. Upon examination of another sample of elastin only such a small amount as 0·15 % of crude tyrosine could be found, which hardly explains the strong Millon reaction obtained with the original material.

It occurred to the author therefore that, in addition to tyrosine, other substances, thus far unknown as decomposition products, also give Millon's reaction. This suspicion was fully verified in the case of elastin. First, it was shown that the basic portion, precipitated from the hydrolysis products by phosphotungstic acid, gave the reaction, even when the precipitate was treated in a manner which precluded any retention of the filtrate. The substance yielding the reaction possesses therefore pronounced basic properties. It was isolated in the following manner.

Air-dry elastin was boiled for 5 hours with about five times the quantity of concentrated hydrochloric acid, and the liquid evaporated upon the water-bath to a syrupy consistence. This was diluted with sufficient water to bring the weight of the liquid to about eight times the weight of elastin used. 50 % phosphotungstic acid was then added and the precipitate after standing for several hours carefully washed or triturated with 5 % sulphuric acid.

This precipitate, as stated in greater detail in the above-mentioned communication[1], was treated with lead hydroxide and after careful washing decomposed with lukewarm baryta solution in the well-known manner. Overheating or great excess of baryta was carefully avoided by adding the baryta solution very slowly in small portions and removing it by suction, when the reaction had remained alkaline for several hours. The residue was treated twice in the same manner. The decomposition took about 2 days and the solution of the basic substances contained only traces of excess of baryta as well as some lead. The lead, after addition of animal charcoal, was removed

[1] R. Engeland and W. Biehler, "New Decomposition Products of Elastin," to be published shortly.

with hydrogen sulphide, and the filtrate was evaporated *in vacuo* to a thin syrup, then boiled with copper hydroxide and filtered. The filtrate was again greatly concentrated, mixed with methyl alcohol until a strong turbidity occurred and set aside at 0° for several days. A considerable precipitate was then noted, partly crystalline, partly consisting of brown flakes. These flakes, on account of their low specific gravity, could mostly be washed off with ice-cold water. The deep blue residue, very slightly soluble in water, was suspended in a little water, and freed from copper by passing hydrogen sulphide through the heated liquid. Upon concentration of the slightly coloured filtrate, long white needles were obtained which were recrystallised from dilute methyl alcohol.

2·782 mg. subst. : 5·997 mg. CO_2 and 1·901 mg. H_2O
2·470 mg. „ : 5·328 mg. CO_2 and 1·536 mg. H_2O
3·426 mg. „ : 0·277 cc. N at 708 mm. 11°

Calculated for $C_{16}H_{24}O_5N_2$:	C = 59·22 %,	H = 7·46 %,	N = 8·64 %
Found	C = 58·83 %,	H = 7·65 %,	N = 9·06 %
	58·85 %,	6·96 %	

The values calculated for formulae with 15 C-atoms would also be very close to those found:

Calculated for $C_{15}H_{22}O_5N_2$:	C = 58·03 %,	H = 7·15 %,	N = 8·41 %
$C_{15}H_{20}O_5N_2$	C = 58·41 %,	H = 6·54 %,	N = 9·09 %

However the values found for the copper salt strongly suggest the formula with 16 carbon-atoms. The copper salt forms small, dark blue needles, which are very sparingly soluble in water. The crystals purified by crystallisation from hot water gave the following analysis:

(1) 4·524 mg. subst. : 0·950 mg. CuO
(2) 3·388 mg. „ : 0·691 mg. CuO
4·350 mg. „ : 7·875 mg. CO_2 and 2·240 mg. H_2O*
3·960 mg. „ : 0·275 cc. at 723 mm. and 18°*

Found:	Cu = 16·78 %, 16·30 %	C = 49·39 %,	H = 5·76 %,	N = 7·75 %
Calculated for $C_{16}H_{22}O_5N_2Cu$:	Cu = 16·48 %,	C = 49·74 %,	H = 5 75 %,	N = 7·38 %

* These two analyses were kindly carried out by Dr F. Reindl.

The free substance tastes slightly bitter, and is rather readily soluble in water as well as in acetic (glacial) acid. The aqueous solution upon evaporation on the water-bath is coloured slightly brownish and gives off a flower-like, very characteristic odour. Upon treatment with oxidising agents such as $NaClO_3$ and HCl this odour may be obtained from very small quantities. Upon boiling with concentrated nitric acid a slight yellow coloration is observed, which is intensified when ammonia is added in excess. Millon's reagent produces even in the cold a pink colour, which however is not so intense as with tyrosine. With diazobenzenesulphonic acid in alkaline (Na_2CO_3) solution an orange coloration is observed. An infusion of the conidiophores of two different forms of *Russula* rapidly colours the solution, in presence of air, red with a blue tinge. After several hours the colour changes to bluish brown. The intensity of this colour is seemingly more pronounced than that obtained in the same way with tyrosine. By phosphotungstic acid the substance is precipitated even from very dilute solution. Upon heating

in the melting point apparatus the substance begins to be discoloured at about 200° and sinters at about 220°.

The substance was isolated by the same method from two different hydrolyses. I consider the substance isolated, therefore, as a constant hydrolysis product of elastin and call it *hyphasmine* (from *τὸ ὕφασμα* = the tissue).

The yield amounted to almost 1 %, when very carefully purified elastin was used, and was very much less when another less purified product was examined. Inasmuch as the solution of the substance is decomposed upon heating, it is certain that a considerable portion, originally present, is lost through the method of preparation described above.

With the same method I have tested another material, namely, the membrane of hen's eggs, which also upon hydrolysis yields no tyrosine. However, the substance could not be isolated, nor could I isolate the two new hydrolysis products which were obtained in the investigation jointly carried out with W. Biehler and referred to above.

Concerning the constitution of hyphasmine, one could be tempted to think that we have here a dipeptide, and that tyrosine takes part in its formation. The second component would thus be a hitherto unknown amino-acid—and the compound be characterised by a peculiarly strong resistance to hydrolysis. The composition of the copper salt, however, seems to speak against such an assumption.

CXXIX. OBSERVATIONS UPON THE PREPARATION AND STANDARDISATION OF THE OVARIAN HORMONE.

By FRANK DICKENS, EDWARD CHARLES DODDS and SAMSON WRIGHT.

From the Biochemical Department, Bland-Sutton Institute of Pathology, and the Physiological Department, Middlesex Hospital, London.

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Attempts to demonstrate the presence of an active principle in the ovary date back to the end of the last century, but the first real advance was made by Adler [1912], who published an account of the production of artificial oestrus in animals by the injection of aqueous extracts of whole ovaries. This investigation is entitled to special notice, since it was the first in which a definite attempt at standardisation was made. Adler checked his results by histological examination of the uterine mucosa of his test animals. This work was followed by a series of more elaborate chemical investigations. Iscovesco [1912] extracted whole ovaries with alcohol and obtained a fatty substance possessing activity. Fellner [1913] demonstrated that the active principle was thermostable and soluble in alcohol, ether and acetone.

Very similar results were obtained by Seitz, Wintz and Fingerhut [1914], and later by Herrmann and Fränkel [1915]. The method of the latter workers consists in extraction of the ovaries with alcohol. The solid material is filtered off, and the resulting solution is evaporated to dryness. The residue is extracted with ether, and acetone is added to precipitate the lipoid fraction. The active principle remains after the solvent has been removed by distillation. As much cholesterol as possible is separated from it by fractional crystallisation, after which it is stated that the material can be distilled *in vacuo* at 190°.

Recently the matter has been re-opened by Allen and Doisy [1923], who applied the vaginal smear method of ascertaining the onset of oestrus. Their method for the preparation of the active material is similar to that described by Herrmann and Fränkel. They were able to prepare active material from liquor folliculi, whole ovaries and placenta, but not from corpus luteum. They lay great stress upon the use of liquor folliculi, and appear to incline to the view that the active principle is located mainly in this fluid. In view of the importance of these observations, it was thought that an investigation of these earlier experiments would be of interest.

Method of preparation.

This consists of slight modifications of those employed by Herrmann and Fränkel, and Allen and Doisy. Pigs' ovaries were used as the main source of supply.

The minced tissues were extracted with twice their weight of alcohol, care being taken not to lose any fluid during mincing. After stirring, the mixture was pressed out in a hand press through jean, and the solid material was then extracted in a Soxhlet apparatus with alcohol for 6–9 hours. The alcoholic extracts were then combined and filtered through Chardin papers.

The filtrate was evaporated almost to dryness *in vacuo*, and the residue was taken up in a small volume of alcohol and filtered. On standing, a precipitate of fats and cholesterol separated which was removed by filtration. The solution was again taken down to dryness, and the residue was dissolved in a small quantity of ether, and twice its volume of acetone was added. The precipitated lipins were filtered off. The solid material was then redissolved in ether and again precipitated with acetone. This process was repeated several times. The filtrates were then combined, and evaporated to dryness. The residue was dissolved in a small quantity of alcohol, and some cholesterol was removed by freezing. By repeating this process, the greater part of the cholesterol can be removed. The final product is obtained by distilling off the alcohol *in vacuo*. Some experiments were tried with alternative methods of extraction. The following is an example.

Aqueous extraction.

A small yield of active substance was obtained by mincing 2·78 kg. ovaries (pig) and washing thoroughly with 2·8 l. water. This washing was repeated once more with the same volume of water and the combined washings were acidified until the reddish colour of the washings changed to grey. The washings were concentrated *in vacuo* in a bath at 65° until the volume was approximately 150 cc. Two volumes of alcohol were added and then dilute sodium hydroxide solution until the maximum precipitation occurred. The solution was filtered and the filtrate was once more concentrated to dryness. The solid matter was taken up in hot 95 % alcohol, the solution was filtered and lipins were precipitated by the addition of two volumes of acetone. The precipitate was filtered off and the liquid concentrated. The yield was 4·4 g., the rat-unit of which was 150 mg., corresponding to about nine rat-units from 1000 g. ovaries.

Yields.

The method of preparation already described has been applied to a series of tissues. The ovaries were not specially selected, and were therefore obtained from animals in various stages of the oestrous cycle.

In the following table the yields obtained can be seen.

Table I.

Source	No. of animals	Weight of tissue	Yield g.	Rat unit mg.	Rat unit per kg.	Notes
Pig ovaries, whole ovaries including liquor folliculi	148	1050 g.	1·05	75	14	Without Soxhlet extraction
	70	960	1·14	80	14	
With Soxhlet extraction:						
	92	720	3·69	15	340	
	218	2100	28·8	150	91	
	280	4650	33·9	100	73	
	240	2290	36·0	200	80	
	800	6960	30·35 crude	50	87	Ovaries had very small follicles
			15·85 purified	30	70	
	228	2140	7·6	50	70	
	290	5410	17·75	50	65	
Liquor folliculi only (pig)		250 cc.	0·4	16	100	
		720 cc.	3·7	25	200	
		120 cc.	1·20	50	200	Four ovaries only each with large follicles
Tissue only from above		50 g.	2·25	200	255	
Human ovarian cyst		1100 cc.	Trace	Inactive		
Placenta (human)		385 g.	3·1 crude	200	50	
			0·45 purified	15	85	
Dried pig ovaries		200 g.	13·0	350	150	Dried *in vacuo* at 40°

The yields are similar to those obtained by Allen, Doisy and their co-workers. We have been unable, however, to secure the large yields from liquor folliculi, and from human placenta, described by these workers. The yields obtained by us from liquor folliculi and from the residual ovarian tissue are of the same order. Our findings, therefore, do not support the contention that the active principle is mainly confined to the liquor folliculi. Of a series of commercial preparations tested all proved inactive. One specimen of whole ovaries, dried at 40°, was found to yield 150 rat-units per kg. by the above method of extraction. No active principle could be obtained from hen's eggs. As a negative control, 1 kg. pancreas was worked up by the process. No active material resulted.

Chemical properties.

The active principle consists of a clear, light brown oil, readily soluble in alcohol, ether, acetone, and olive oil. It can be heated to 200° without loss of activity. It withstands saponification only to a limited extent, as can be seen from the following experiments.

1 g. sodium was dissolved in 20 cc. absolute alcohol, and the solution added to 10 cc. of an alcoholic solution of the active principle containing 6·28 g., the rat-unit being 40 mg. The mixture was heated on a boiling water-bath, in an atmosphere of hydrogen for 40 minutes. After cooling, the contents of the flask were poured on to powdered ice, and the resulting liquid was thoroughly extracted with ether. The extracts yielded 0·4 g., which was found to give a positive reaction in rats in doses of 40 mg. The fatty acids obtained

by acidification of the aqueous layer were found to be inactive physiologically, even in doses of 0·5 g. In another experiment, where the reaction was allowed to proceed for 3 hours, the product was quite inactive. It appears therefore that the activity is slowly destroyed under these conditions. Attempts at distillation have up to the present been unsuccessful, owing to excessive frothing.

Purification by digitonin.

The cholesterol-containing substance obtained by the usual alcoholic extraction process (3 g. = 37 rat-units) was dissolved in 60 cc. hot 95 % alcohol. A solution of digitonin (1 g.) in hot 90 % alcohol (100 cc.) was added, and after 1 hour the fine crystalline precipitate was filtered off, and the filtrate was concentrated to dryness on the water-bath. The residue was taken up by ether, the extract filtered and the ether removed. The clear, pale yellow, oily residue (0·75 g.) no longer gave the Liebermann-Burchardt reaction for cholesterol. By this treatment with digitonin the rat-unit was reduced from 80 mg. to 25 mg. The above yield therefore represents approximately 30 rat-units, showing that there is little loss in this method of purification.

Method of testing extracts.

This depends on the work of Stockard and Papanicolaou [1917] with the guinea-pig, which was extended to the rat by Long and Evans [1922]. It was demonstrated that a definite oestrous cycle exists in these animals, and that the various stages of the cycle can be accurately determined by examining smears from the vagina. Four stages of the oestrous cycle in the rat can be recognised: pro-oestrus, oestrus, post-oestrus and post-ovulation. The total duration of the cycle is 96 hours. The changes found in the smears depend on proliferation of the vaginal epithelium and keratinisation of these cells, proliferation of the uterine mucous membrane and migration of leucocytes. In cases of doubt, the animal may be killed and the genital organs examined both with the naked eye and microscopically.

Ovarian extracts were standardised by the method of Allen and Doisy, *i.e.* by determining the minimum amount which will bring about artificial oestrus in ovariectomised adult animals. Rats were used almost exclusively. The extracts were injected subcutaneously, dissolved in olive oil or suspended in weak bicarbonate solution. If the extract was active, oestrus appeared sometimes after 24 hours and usually within 36 hours of the time of injection.

The extracts containing much cholesterol were not easily absorbed and large "granulomata" appeared at the site of injection. When extracts freed from cholesterol were employed, practically no nodules resulted or they were much less marked. The total dose of extract to be given can be administered at one injection as absorption is quite slow. The same results were obtained from one dose as from a spread-out series of small doses which amounted to the same total. The responsiveness of the animals was found to wear off with

the lapse of time after ovariectomy and larger doses became necessary for the production of oestrus. Thus 12 mg. of a liquor folliculi extract produced definite oestrus in a freshly spayed rat, while 50 mg. produced only slight pro-oestrus in a rat spayed 3 months previously. It must be remembered that 3 months corresponds to 30 oestrous cycles in the rat, which is equivalent to $2\frac{1}{2}$ years in the human female. After 3 months the genital organs of ovariectomised rats are very thin and resemble fibrous cords. The effect of the preparation was also tested upon a female monkey: 100 mg. were injected subcutaneously, and within 3 days typical menstruation commenced. That this response was not the onset of a normal period was excluded by previous observations upon the animal. In a series of experiments in which the extract was administered by the mouth to ovariectomised rats, oestrus could not be induced even when very large doses were given.

Depressor effect.

Intravenous injection of active ovarian extract dissolved in olive oil was carried out on anaesthetised animals. As controls, solutions of cholesterol in olive oil were employed. As will be seen from Fig. 1 A the control injections produced a slight irregularity of the blood pressure in doses from 1–4 cc. The ovarian extract had a marked depressor effect; a fall of 24 mm. Hg resulting from injection of 0·1 cc. (Fig. 1 B) and a fall of 46 mm. Hg from a dose of 0·2 cc. (Fig. 1 C). With a dose of 1 cc., the depressor effect was more prolonged (Fig. 1 D). No marked effect on respiration was noted.

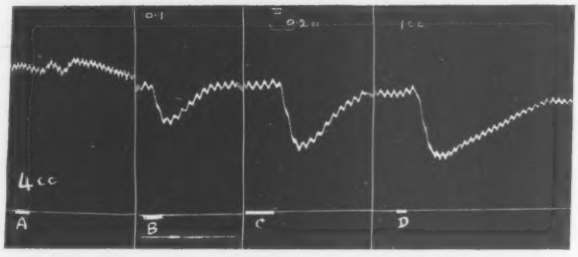

Fig. 1. Cat. Ether. Blood pressure. At A, 4 cc. of emulsion of cholesterol in olive oil injected. At B, ovarian extract in olive oil 0·1 cc. At C 0·2 cc. At D 1 cc. Time in seconds.

One experiment was carried out in which daily injections of ovarian extract were administered to a rat over a period of 14 days. One rat-unit was contained in each 1 cc. of the solution used and the injections were given daily in doses of 1 cc.

Smears from the vagina were studied. Oestrus appeared normally after 36 hours. Typical cornified cells were present till the sixth day of the experiment. No leucocytes or fibrin were found. On the sixth day a characteristic pro-oestrus smear appeared followed by oestrus cells on the seventh day.

Further pro-oestrus smears were obtained on the tenth day and succeeded by oestrus cells. On the fourteenth day the animal was killed. The uterine horns and body were distended and considerably congested. The vagina was widely dilated, *i.e.* typical oestrus conditions were present. Apparently the effect of continuous injection of the ovarian principle was greatly to prolong the period of oestrus. The post-ovulatory stage (appearance of leucocytes) was not noted and there was no resting stage; but oestrus lasting as long as 5 days was followed by a new pro-oestrus and then further oestrus. Throughout the period of the experiment the vaginal fluid had the characteristic watery appearance with small opaque granules like small rice grains as is seen in natural oestrus.

Interaction between insulin and the active principle.

That the onset of menstruation disturbs the carbohydrate metabolism to some extent has been pointed out by many workers.

Thus it is known that the advent of the period in a diabetic patient is often accompanied by an increase in the blood and urine sugar content, although the diet remains constant.

This suggested that there might be some interaction between insulin and the active principle of the ovary. Since the oestrous cycle has been fully worked out in mice, and their response to insulin is also well known, it was decided to employ these animals to test the point.

In the following tables can be seen the effect of varying doses of insulin upon mice not in oestrus, mice in natural oestrus, and mice in which artificial oestrus has been induced by injection of the ovarian principle.

A similar experiment was performed upon rabbits, the results of which appear in Table V.

Effect of insulin on female mice.

The mice were maintained at a temperature of 28°.

Table II. *Normal mice, not in oestrus.*

Insulin solution: a clinical solution diluted 1 in 300.

Dose cc.	Result
0·5	Completely comatose 1 hr. 25 mins. after injection. Revived by glucose
0·4	Comatose 2 hrs. after injection. Revived by glucose
0·3	Slightly sleepy condition 2 hrs. after injection
0·2	Nil
0·1	Nil

Table III. *Normal mice in natural oestrus.*

The same solution of insulin was injected as in Table II.

Dose cc.	Result
0·5, 0·4, 0·2, 0·1	No effect visible up to 5 hrs later, when the mice were fed

Table IV. *Mice in artificial oestrus induced by injection of ovarian extract (from pig's ovaries).*

Insulin solution was a 1 in 100 dilution of a clinical solution.

Dose cc.	Mice in artificial oestrus	Non-oestrous controls
0·4	No apparent action	Comatose in under 2 hrs. and recovered on administering glucose
0·2		

Table V. *Effect of ovarian extract on the reaction to insulin of normal rabbits.*

Rabbit no.	Insulin weight mg.	Blood-sugar		
		Resting	1 hour	2 hours
Before injection of ovarian extract:				
9	0·5	110	70	40
12	1·0	98	40	29
14	1·5	120	33	19
After five injections 0·1 g. of ovarian extract at six hourly intervals:				
9	0·5	110	97	89
12	1·0	125	79	54
14	1·5	105	50	41

These experiments demonstrate the fact that the active principle of the ovary possesses the property of inhibiting the action of insulin. It is interesting to note that animals in natural oestrus respond in the same manner as those in which artificial oestrus has been induced.

Summary.

1. A physiologically active principle can always be extracted from the ovary by means of alcohol, as a number of earlier workers have found.

2. The substance is an oil, soluble in alcohol, ether and acetone, and can be obtained free from cholesterol. Experiments are described showing its behaviour towards sodium ethoxide on saponification.

3. The method of standardisation is discussed, together with the general physiological properties.

4. By means of a series of injections an ovariectomised rat was kept in a state of continuous oestrus for a period of 14 days.

5. Administration by the mouth proved to be ineffective.

6. The extract was found to have a marked depressor action.

7. It was found that the substance inhibited the blood-sugar reducing action of insulin.

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CXXX. FACTORS INFLUENCING THE ACTION OF PANCREATIC LIPASE.

By BENJAMIN STANLEY PLATT and EDWARD ROMER DAWSON.

From the School of Medicine, University of Leeds.

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In the course of a determination of the action of pancreatic lipase on certain esters, it was necessary to find a convenient method of preparing the enzyme in an active and pure condition, and to investigate the influence of certain factors with a view to obtaining the optimal conditions of action.

Method of Preparation.

The methods which have been devised for utilising the enzyme in the organ may be divided into three main groups: (*a*) the whole pancreas may be used, or a simple press juice obtained from it, (*b*) the enzyme may be extracted from the fresh gland with suitable solvents and the extract used in the crude state or subjected to further purification, (*c*) the pancreas may be treated so as to obtain a solid preparation containing the lipase. The solid obtained by this last method can be worked up to yield an enzyme in a high degree of purity as in the method of Willstätter and Waldschmidt-Leitz [1923].

Most of the previous work on the action of pancreatic lipase has been carried out with an enzyme preparation containing the natural activating substances. A determination of the effect of added substances on the activity of such a preparation is obviously of little value. Accordingly, we employed a method suggested by Rosenheim [1910] and developed by Umeda [1915]. The powder obtained by this method is claimed to be free from water-soluble substances, including enzymes, and organic substances soluble in alcohol, acetone and ether. It is found that practically all the amylase originally present in the extract is removed in the preparation of the powder, which however has still considerable tryptic activity. A sample of dried pancreas powder has been prepared by the method recommended by Willstätter and Waldschmidt-Leitz [1923], and the enzyme content of the two preparations compared under identical conditions. Lipase is present to the same extent in both samples, whilst in the preparation obtained by Willstätter's method, amylase is present, and the trypsin content is greater than in the powder prepared by Umeda's method.

In agreement with Virtanen [1924], we have found that the amount of lipase extracted by glycerol varies with the time allowed for extraction.

When, however, the gland is reduced by further mincing to a fine pulp, a few hours suffice for complete extraction of the enzyme. Willstätter and Waldschmidt-Leitz [1923] object to the use of alcohol in obtaining a solid preparation of the pancreatic enzymes. They find that treatment with alcohol causes an appreciable diminution in the activity of the lipase. We find that alcohol has no influence on the lipase in the powder obtained by Umeda's method, probably because this substance has already been washed with alcohol. We would suggest that the loss in activity noticed by Willstätter is due to the removal of an activating substance soluble in alcohol. In fact, Umeda has found that the so-called "co-enzyme" of lipase is made up of two fractions, one of which is soluble in alcohol. It is noteworthy, too, that Foster and Woodrow [1924] obtain a stable mixture of pancreatic enzymes by desiccating the organ with alcohol. Also, Vollmer, Schmidt and Serebrijski [1924], from the results of experiments on the antilipolytic effect of solutions of iodine in alcohol, state that the inhibition of these solutions is not due to the alcohol.

Estimation of Activity.

The activity of the lipase obtained from the pancreas has been determined in the following experiments by estimating the amount of acid liberated from ethyl butyrate or from olive oil. When the latter substance was used as the substrate, the technique developed by Willstätter and his collaborators was adopted. No antiseptics were added to the digestion mixtures, as Umeda [1915] and Long, Hull and Atkinson [1917] found this precaution unnecessary in the case of olive oil, and we have found in our work that ethyl butyrate has a pronounced inhibitory influence on bacterial growth.

A number of workers have directed attention to the inconsistent results obtained when a series of digestion mixtures are not all shaken to the same extent [cf. Bradley, 1909; Pekelharing, 1912; Haley and Lyman, 1921; and Willstätter, Waldschmidt-Leitz and Memmen, 1923]. We have used a device similar to that described by Armstrong and Gosney [1913], viz. shaking the test mixtures while in the incubator. The shaking would appear to be essential in this case to eliminate any discrepancies which might arise due to such physical causes as diffusion. Continued shaking was found to have no deleterious effect on the action of lipase on ethyl butyrate. On the contrary, a series of mixtures shaken for different periods up to 3 hours, were (at the end of 3 hours) hydrolysed to a degree proportionate to the time of shaking.

In the experiments on the activity of lipase at different hydrogen ion concentrations, we found that the amount of action could be easily and accurately determined by titrating a sample of the digestion mixture to the initial p_H with CO_2-free alkali, using a suitable indicator. Due regard was given to the concentration of indicator and to compensation for opalescence and colour of test solutions.

Influence of Hydrogen Ion Concentration.

Little importance can be attached to the results of earlier work on the action of lipase owing to the failure to appreciate the necessity for regulating the reaction of the digestion mixture. Especially is this the case for the work on the influence of added substances. As Willstätter and his co-workers have shown in recent papers, a substance may have quite different effects according as the medium is acid or alkaline.

Under certain conditions, enzymes are considered to display maximal activity at a definite hydrogen ion concentration, which is termed the "optimum p_H." This property is regarded by many as characteristic of the particular enzyme. In seeking for the conditions for maximal activity of lipase it is necessary to determine and maintain this optimum p_H. Peculiar difficulties arise in the course of lipolysis due to the liberated acid changing the p_H of the medium. After various attempts had been made to eliminate the effect of the acid formed, we found that it was not possible to keep the p_H constant; but by using buffer solutions of suitable concentrations, the amount of change could be restricted. By increasing the concentration of the buffer solution to a point consistent with the accurate determination of the end point by titration, the range of p_H over which the enzyme was allowed to work could be reduced to as little as 0·2.

There are a number of values given in the literature for the optimum p_H of pancreatic lipase. Rona [1911] determined the action of lipase in different organ extracts at p_H 7·5, in phosphate buffer mixtures. Davidsohn [1912] found a reaction optimum of p_H 8·0 and in the following year [1913] he gave the value p_H 8·5. Further work of Rona and Bien [1914] suggests that there is an optimal zone over which the lipase of a pancreatic extract is most active, namely, from p_H 8·4 to p_H 9·0. Umeda [1915] gives a value of about p_H 7·4 for the action of the lipase in his extract of pancreas on olive oil in phosphate buffer solutions. With a saline extract of pancreas or with a pancreatic fistula secretion, Rona and Pavlovic [1923] obtained for the optimum p_H a value of 6·95 to 8·0. Some of the results, when represented by curves obtained by plotting activity against increasing p_H, do not show an optimal point or even a zone of maximal activity, but simply that a limiting value is being approached. The recent values obtained by Rona and Pavlovic, however, definitely prove that at p_H 3·7 there is no lipase activity and that above p_H 8·0 there is a distinct falling off in activity. Willstätter prefers to work with a buffer solution containing ammonia and ammonium chloride at a p_H of 9·2 at 18° when using olive oil as substrate, and a similar buffer solution at p_H 8·6 when tributyrin is the substrate. Anrep, Lush and Palmer [1925] carry out their lipase estimations in a phosphate buffer solution at p_H 7·8 and allow the reaction to proceed until the p_H is not less than 7·0.

Determination of optimum p_H.

The results of typical experiments carried out to determine the optimum p_H for pancreatic lipase are given in the curves in Fig. 1. Mixtures were made up as indicated in 50 cc. flasks of Jena glass fitted with rubber stoppers. All the solutions and vessels were previously warmed up to the reaction temperature. In all cases the substrate was added last to the digestion mixture. These were shaken mechanically in an incubator kept at 37°. To stop the reaction the flasks were plunged into ice-water for 10 minutes before titration. The results given are the mean of duplicate experiments, corrected for suitable controls, and are expressed in cc. CO_2-free alkali required to restore the initial p_H estimated colorimetrically and at room temperature. The buffer mixtures used are given by Clark [1922], Kolthoff [1925] and Palitzsch [1915].

Exp. 1. Mixture: 24·0 cc. KH_2PO_4—NaOH buffer solution (Clark).
0·5 cc. ethyl butyrate.
1·0 cc. suspension of 5 mg. of enzyme in water.

Incubated 3 hours at 37°, then titrated with $N/20$ NaOH using either bromothymol blue, phenol red or thymol blue as indicator.

The titration values are for 10 cc. of the reaction mixture (see curve, Fig. 1).

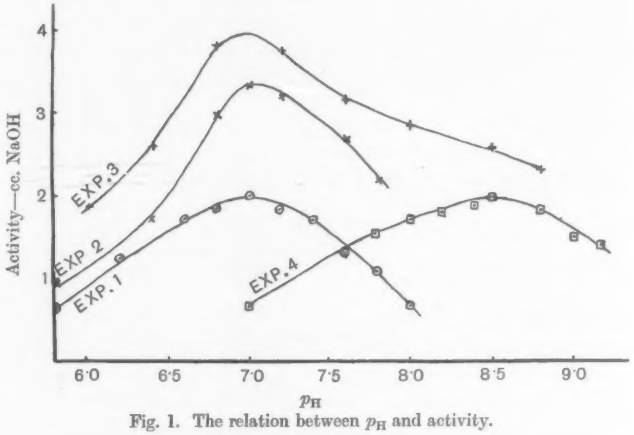

Fig. 1. The relation between p_H and activity.

Exp. 2. Mixture: 10·0 cc. phosphate buffer solution (Clark).
0·5 cc. ethyl butyrate.
1·0 cc. suspension containing 5 mg. enzyme.
Alkali used: $N/10$ NaOH.
Other conditions as in previous experiment (see curve, Fig. 1).

Exp. 3. Conditions as in previous experiment, but 10 cc. of Kolthoff's borax-phosphate buffer used instead of the phosphate solution above (see curve, Fig. 1).

Exp. 4. Conditions as in previous experiments.

Mixture: 46 cc. borax-boric acid-NaCl buffer solution (Palitzsch).
1 cc. ethyl butyrate.
2 cc. suspension containing 10 mg. enzyme.

The results are shown in the curve, Fig. 1.

It will be seen from these results that the optimal activity of pancreatic lipase in phosphate and phosphate-borax buffer solutions with ethyl butyrate as substrate occurs at about p_H 7·0, whilst in Palitzsch's buffer mixture there is a zone of maximal activity about p_H 8·5.

The following experiments were carried out with a crude glycerol extract of pig's pancreas. 0·5 cc. of the extract, which had been strained twice through muslin, was diluted with 5 cc. of phosphate buffer solutions of various hydrogen ion concentrations, and 3 cc. of water, 0·5 cc. of ethyl butyrate and 0·5 cc. of toluene were added. After 3 hours' shaking at 37° the mixture was titrated to a pink colour with $N/10$ NaOH, using phenolphthalein as indicator.

Exp. 5.

p_H	Mean titration value (corr.) cc.
5·8	3·53
6·2	3·94
6·8	4·86
7·0	5·20
7·4	5·35
7·6	5·51
7·8	6·55

The experiment was repeated with the same quantities of extract, ester and toluene in 25 cc. of the buffer mixtures. 10 cc. portions were titrated as in the preceding experiment after 3 hours' incubation.

Exp. 6.

p_H	Mean titration value (corr.) cc.
6·2	3·4
7·0	5·6
7·4	5·6
7·6	5·75
7·8	6·1
8·0	6·25

From a consideration of the foregoing results, it is easy to understand why anomalies occur in the findings of different workers. The substances accompanying the lipase in the crude glycerol extract appear to protect the enzyme from destruction in the more alkaline media.

The effect on lipase action of a preliminary treatment with buffer solutions of different p_H.

The optimum p_H of pancreatic lipase may be considered as the outcome of two opposing tendencies. With increase in alkalinity an increase in hydrolytic activity takes place, but at the same time, the enzyme is less stable.

This idea is suggested by the work of Noyes, Suguira and Falk [1923], and is borne out by the results of the following experiments.

10 mg. of enzyme were added to 15 cc. of phosphate buffer solution (Clark) of various p_H. This suspension was shaken in the incubator for 24 hours at 37°. Each sample was then adjusted with the calculated amounts of alkali, phosphate solution or water, so that the final p_H was 7·6 and the salt concentration of the mixtures was the same in all cases. 0·5 cc. of ethyl butyrate was added and the mixture shaken again in the incubator for 24 hours, when 10 cc. aliquots were titrated with $N/10$ NaOH to p_H 7·6, using phenol red as indicator.

Exp. 7.

p_H	Mean titration value (corr.) cc.
6·6	3·35
7·0	3·25
7·2	3·29
7·4	3·33
7·6	2·88
7·8	2·35
8·0	1·90

A similar experiment was carried out using Clark's borax-boric acid-KCl buffer mixtures. All the mixtures, after 24 hours' contact with the respective solutions, were adjusted to p_H 8·6, and the activity of the enzyme estimated by its action on ethyl butyrate for 24 hours.

Exp. 8.

p_H	Mean titration value (corr.) cc.
7·8	1·92
8·0	2·19
8·2	1·66
8·6	1·42
9·2	1·40

A study of the relationship between p_H and lipase activity in the various buffer solutions, and of the effect of a preliminary treatment with buffer solutions of different p_H before estimating the activity, suggests that the "buffer value" of the mixtures determine to some extent the form of the optimum curves. The position of the optimal zone when Palitzsch's solutions are used coincides with the position of the maximum buffer value. Also, when the buffer value of the phosphate solutions is increased on the alkaline side by the addition of borax, as in the Kolthoff buffer mixtures, an increased activity is observed.

The Effect of Added Substances on the Action of Pancreatic Lipase.

Substances added to lipase may affect its action by establishing a favourable p_H, by maintaining this p_H because of buffer value, or by some specific influence, probably chemical in nature, on the enzyme. In an experiment

in which lipase was introduced into a solution of ethyl butyrate at a p_H suitable for lipolytic action, no lipase action could be detected by comparing the rate of change of p_H of this mixture with that of a similar solution of ethyl butyrate containing no enzyme.

The enzyme for this experiment was obtained from the powder prepared by Umeda's method, and was freed as far as possible from accompanying substances by digestion in 90 % glycerol and subsequent filtration through a hardened filter paper, with suction. A clear solution was thus obtained containing a considerable amount of lipase. This was added to a solution of pure ethyl butyrate in CO_2-free distilled water. A control experiment was set up in which the same amounts of ester solution and glycerol were present. Samples were withdrawn at frequent intervals and the p_H estimated colorimetrically. Special precautions were taken to prevent contamination from the vessels used and from CO_2 in the atmosphere. It was found that the time taken for the change in p_H from 7·8 to 7·05, using the same indicator, phenol red, was identical in both experiments. Apparently some activating substance must be added to the enzyme before it can function even at a suitable hydrogen ion concentration.

The influence of phosphates.

In determining the most suitable concentration of buffer solution required to restrict the amount of change in p_H during the course of lipolysis, it became necessary to investigate the effect of various concentrations of phosphate solutions on lipase activity at a definite initial p_H. The following are typical of the results obtained.

A series of mixtures was prepared containing 20 mg. of enzyme in 10 cc. of phosphate buffer solution and 0·1 cc. of ethyl butyrate. Various salt concentrations were used, the initial p_H being 7·6. After shaking for 1 hour in the incubator, the mixtures were titrated with $N/20$ NaOH to p_H 7·6, using phenol red as indicator.

Exp. 9.

Molar concentration of phosphate	Mean titration value (corr.) cc.
—	0·44
0·005	0·99
0·01	1·2
0·02	1·48
0·025	1·57
0·03	1·82
0·04	2·00
0·05	2·23
0·075	2·08

The range of concentration was increased in the following experiments. Buffer mixtures at p_H 7·6 and 7·2 were made up, the concentrations of phosphate being 0·2M. These solutions were diluted to give solutions of the required salt content. The mixtures contained 25 cc. phosphate solution, 0·5 cc. ethyl butyrate and 1 cc. suspensions containing 10 mg. of enzyme.

10 cc. aliquots were titrated at the end of 3 hours' incubation with $N/20$ NaOH, using phenol red as indicator for the first and bromothymol blue for the second series. 10 cc. portions were titrated again after the reaction had proceeded for a further 21 hours.

Exp. 10. The results are shown in the curves of Fig. 2.

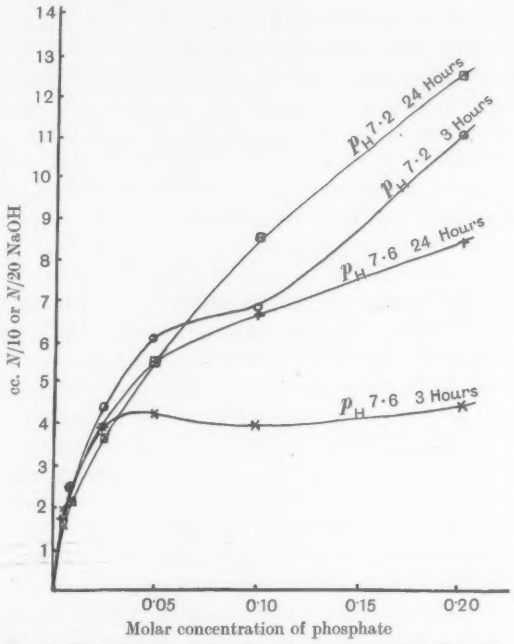

Fig. 2. The influence of phosphate concentration (see Exp. 10).

The experiment was repeated using different phosphate concentrations.

Exp. 11. p_H 7·6.

Concentration of phosphate M	Mean titration value (corr.) 3 hours cc.	24 hours cc.
0·025	1·77	5·07
0·05	1·91	7·36
0·075	1·68	7·32
0·1	1·63	7·96
0·15	1·72	8·72
0·2	1·75	9·62

It will be seen from these results that Clark's phosphate buffer solutions of the usual concentration (0·05M KH_2PO_4) are suitable media for carrying out lipase tests on ethyl butyrate as substrate.

A straight line relationship between the concentration of the phosphate solution and the activity of the lipase would be anticipated if only a buffer

effect were involved. The relationship is no doubt modified by a specific salt effect similar to that which is found in the case of synthetic bile salts. When the amount of lipase action is increased by carrying out the reaction at a p_H nearer the optimum value, or by lengthening the reaction period, a closer approximation to this relationship is observed.

Solutions of sodium α- and β-glycerophosphates, when compared with a solution of potassium phosphate of the same molar concentration and at the same initial p_H, were found to activate lipase to a greater extent, when tested under the same conditions. The two glycerophosphates were identical as regards their activating powers, thus suggesting that the phosphate ion is responsible for the activation. The reason for the different effects of these salts probably lies in their buffer values, which is found to be greater for the glycerophosphates than the inorganic phosphate at the p_H employed. In view of the above results, the objection raised by Noyes, Suguira and Falk [1923] to the use of buffer solutions in the estimation of lipase cannot be sustained.

The adjuvant action of phosphates has been clearly demonstrated by Umeda [1915]. Also, Long, Hull and Atkinson [1915] adduced evidence that the salts present in the pancreas are mainly phosphates. One function of these phosphates is evidently to activate the lipase of the pancreatic juice. Willstätter, Waldschmidt-Leitz and Memmen [1923], by adding calcium chloride, albumin and an ammonia-ammonium chloride buffer mixture, claim to have produced a medium in which lipase from any source is completely activated. We find that the addition of small amounts of phosphate increases the action of pancreatic lipase, even when the system of activators recommended by Willstätter is present. Thus, in one of a number of similar experiments on the effect of small amounts of phosphate solution on the action of lipase on olive oil, we find, by using Willstätter's technique, that 4·05 cc. 0·3*M* alcoholic KOH are required to neutralise the acid formed from 2·5 g. olive oil. Increased amounts of the same alkali are required, 5·8, 4·8 and 4·7 cc., when 0·25, 0·5 and 1·0 cc. respectively of the elution mixture, used in the process of the purification of lipase described by Willstätter and Waldschmidt-Leitz [1923], are also present. The above titration figures are the mean of the results of duplicate experiments, corrected for the necessary control. The enzyme powder used in this experiment was prepared by Willstätter's method and was found to have an activity of the same order as that given in this author's paper. When in the later stages of purification of lipase Willstätter tested for the amount of enzyme, the estimations were carried out in the presence of amounts of elution mixture similar to those used above. The lipase values would therefore not be comparable with the initial values obtained in mixtures not containing phosphate.

The influence of bile salts.

It is remarkable that the results of a number of workers, de Jonge [1917], Orbeli and Tetyaeff [1916] and Terroine [1920], on the influence of various

concentrations of bile salts on the activity of pancreatic lipase show the same relationship as we have observed for the effect of increasing amounts of phosphates. Anrep, Lush and Palmer [1925] have attributed to bile salts a specific chemical influence, distinct from their surface tension properties, which they hold in common with substances such as caprylic alcohol and saponin. We would suggest that the specific chemical effect, which both phosphate and bile salts can exert, is partly responsible for the connection observed between salt concentration and enzyme activity. It is probable also that the same factor causes the shift in optimum temperature of lipase in the presence of bile salts. Terroine [1920, p. 199] gives figures which show that an elevation of the optimum temperature of lipase in pancreatic juice occurs when bile salts are present, but he does not comment on this fact. In some preliminary work on this point, we have obtained results similar to those of Terroine.

The effect of albumin and edestin.

Emphasis is laid by Falk [1921] on the protein structure of lipase. The preparations we have obtained at different times contain amounts of nitrogen ranging from 8·5–12·3 %. Moreover, the activity of a definite amount of each sample is directly proportional to the amount of nitrogen in the powder. We do not take these results as evidence of the protein nature of the enzyme, but rather that the enzyme is associated with a protein substance and has been freed from impurities to varying extents, depending upon the efficiency of the washing. The accompanying protein, however, largely determines the properties of the enzyme. Falk [1915] has stated that he has been able to separate the ester-hydrolysing enzymes of castor bean into a "lipase" and an "esterase," the former being the globulin fraction, the latter an albumin-like substance. The following experiments were performed to show that the enzyme is not necessarily a protein, but it can manifest so-called "esterase" or "lipase" properties according as the same enzyme material is accompanied by one or other of the two proteins.

A clear, filtered glycerol extract of dried pancreas was used as the source of lipase. The albumin was crystalline egg albumin (Boots), and the globulin was edestin. The mixtures prepared were:

(1) 18 cc. distilled water.
0·5 cc. glycerol extract of enzyme in 2 cc. of water.
0·5 cc. ethyl butyrate.

(2) 14 cc. distilled water.
4 cc. solution containing 10 mg. albumin.
0·5 cc. glycerol extract in 2 cc. water.
0·5 cc. ethyl butyrate.

(3) As (2) but 20 mg. albumin.

(4) As (2) but 10 mg. edestin.

(5) As (2) but 20 mg. edestin.

(6), (7), (8), (9) and (10) are comparable with the above, except that 2·5 g. of olive oil are used as substrate instead of ethyl butyrate. The results are expressed in terms of $N/10$ NaOH required to neutralise the butyric acid liberated in the first series after 18 hours' shaking in the incubator at 37°. In the experiments with olive oil, the titration was carried out in alcohol-ether solution with 0·397N alcoholic KOH. Phenolphthalein was used as indicator in both cases. The values recorded are the mean of duplicate determinations corrected for the necessary control. The results of two experiments, using different glycerol extracts, are given in the following table.

Exp. 12.

Mixture. Enzyme and substrate		Substrate: Ethyl butyrate Exp. 1 cc.	Ethyl butyrate Exp. 2 cc.		Olive oil Exp. 1 cc.	Olive oil Exp. 2 cc.
Plus water	(1)	0·43	0·48	(6)	1·6	0·99
Plus 10 mg. albumin	(2)	1·9	1·2	(7)	0·45	0·57
Plus 20 mg. albumin	(3)	2·5	1·85	(8)	0·49	0·83
Plus 10 mg. edestin	(4)	1·15	0·75	(9)	1·8	1·33
Plus 20 mg. edestin	(5)	1·4	1·0	(10)	1·8	1·90

Clearly from these results, the same enzyme, associated with albumin, hydrolyses a simple ester such as ethyl butyrate more easily than a typical glyceride, olive oil, which however is decomposed to a greater extent than ethyl butyrate when the lipase is allowed to work in association with edestin. It seems that Falk's conclusions were based on the isolation of the two different proteins, on which the same enzyme was adsorbed, rather than a separation of two ester-splitting enzymes.

The action of trypsin on lipase.

Conflicting evidence is found in the literature on this subject. We find that Lewkovitsch and Macleod [1903], Mellanby and Woolley [1914], Terroine [1920] and Mellanby [1925] state that trypsin destroys lipase. On the other hand, Shaw-Mackenzie [1915] and de Souza [1916] find that lipase and trypsin may be preserved together in extracts of pancreas containing more than 30 % glycerol. The glycerol is supposed to inhibit the action of trypsin on lipase, and on dilution the protective action of the glycerol is removed. We have found, however, that in solutions containing up to 30 % glycerol it is possible for trypsin to decompose caseinogen. Further, if trypsin is allowed to act in solutions containing active lipase, the loss of lipase is not so marked if due regard is given to the destruction of protein activating substances of the lipase. Willstätter and Memmen [1924] have remarked on the instability of lipase in aqueous extracts of pancreas. They find that the lipase is destroyed even when all the trypsin is removed from solution.

The following experiments were conducted to determine the nature and extent of the supposed destructive action of trypsin on lipase. Solutions of

lipase were subjected to the action of trypsin. The amount of lipase in these mixtures was estimated after intervals by the action on olive oil, in the presence of protein activators, or on ethyl butyrate.

Exp. 13. A mixture (A) was prepared containing:

5 cc. phosphate buffer solution at p_H 7·8.
200 mg. pancreas powder (Willstätter).
20 cc. neutral "liquor pancreaticus" (Benger).
25 cc. water.

A second mixture (B) was prepared in which the "liquor pancreaticus" had been boiled before addition.

These mixtures were maintained at a temperature of 37°. All the solutions and vessels used were previously warmed up to this temperature. To estimate the amount of lipase, 4 cc. of each mixture were withdrawn and added to 10 cc. of phosphate buffer solution at p_H 7·8, 0·5 cc. of ethyl butyrate then being added. The estimations were carried out in duplicate with the necessary controls. After incubating for 1 hour, the amount of $N/10$ NaOH required for neutralisation of the acid formed was determined in the usual manner.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	6·65	5·85
1	4·25	5·6
3	1·55	2·75

Exp. 14. The following results were obtained from a similar experiment, using the mixture (A):

100 cc. phosphate buffer at p_H 8·2.
200 mg. pancreas powder.
30 cc. neutral "liquor pancreaticus."

As before, mixture (B) contained boiled "liquor pancreaticus." For the lipase test, 10 cc. of the mixture were withdrawn, 0·5 cc. of ethyl butyrate added and the estimation carried out as above.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	5·8	5·95
1	2·2	4·45
2·5	0·8	2·5
4·5	0·5	1·18

Willstätter's technique for the estimation of the amount of lipase was used in Exps. 15 and 16. For each test, 2 cc. of mixture were withdrawn. 0·398 N alcoholic KOH was used for titration of the acid liberated from the olive oil.

Exp. 15. Mixture (A) contained:

1·25 cc. phosphate buffer solution at p_H 7·8.
50 mg. pancreas powder.
5 cc. neutral "liquor pancreaticus."
6·25 cc. water.

In the case of mixture (B), the "liquor pancreaticus" had been boiled before addition. The following results were obtained:

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	2·6	3·15
3	1·1	1·65
24	0·22	0·23

Exp. 16. In this experiment, mixture (A) contained:

2·5 cc. phosphate buffer solution at p_H 7·8.

100 mg. pancreas powder.

20 cc. neutral "liquor pancreaticus."

2·5 cc. water.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	4·94	5·16
2	3·04	3·34
4	2·0	2·78

The amount of trypsin in the mixtures containing unboiled "liquor pancreaticus" was determined and was found to be 4–6 times as great as in the mixtures which contained boiled "liquor pancreaticus."

Experiment showed that there is no measurable tryptic decomposition of the small amounts of albumin present when Willstätter's technique is used.

From the results of the preceding experiments it is seen that there is an appreciable difference in the lipase contents of the various mixtures if no allowance is made in the estimations for the loss by tryptic action of protein activating substances of the lipase. When large amounts of trypsin are present and the lipase estimations are carried out by the ethyl butyrate method, the diminution in lipase content is very pronounced (Exps. 13 and 14). If, however, albumin is added to compensate for protein losses by tryptic action, as in the tests by Willstätter's method, the rate of loss of lipase is not very different from that of the solutions to which no further amounts of trypsin had been added (Exps. 15 and 16).

The effect of formaldehyde.

De Souza [1916] has found that formaldehyde in concentrations of 1 : 1000–1 : 500 does not destroy lipase. Palmer [1922] has shown that solutions containing 0·1–0·05 % of formaldehyde have no effect on lipase action. These results are important in view of the theory of the protein nature of lipase. We have obtained the following results which are in agreement with the foregoing.

Mixtures were made up containing:

20 cc. phosphate buffer solution at p_H 7·2.

5 cc. formaldehyde solution.

5 mg. enzyme powder in 1 cc. buffer solution.

0·5 cc. ethyl butyrate.

Test mixtures, in duplicate, with necessary controls, were shaken in the incubator for 3 and 24 hours at 37°. The results are expressed in cc. of $N/10$ NaOH required to restore the p_H of 10 cc. to the initial value, using bromothymol blue as indicator.

Exp. 17.

Concentration of formaldehyde	Mean titration value (corr.) 3 hours cc.	24 hours cc.
0	7·25	10·75
1 : 2500	6·16	10·4
1 : 2000	6·06	10·4
1 : 1500	5·73	9·9
1 : 1000	5·82	9·8
1 : 500	5·32	8·4
1 : 100	2·64	6·3

An experiment carried out in the same manner gave the following results:

Exp. 18.

Concentration of formaldehyde	Mean titration value (corr.) 3 hours cc.	24 hours cc.
0	3·7	8·8
1 : 2000	3·02	7·1
1 : 1000	3·05	7·7
1 : 500	3·29	7·6
1 : 100	1·35	5·2
1 : 50	0·90	2·9

For concentrations of formaldehyde less than 0·2 %, especially for the longer reaction periods, the formaldehyde appears to have but little inhibitory influence. If the enzyme had the structure of a typical protein the effect of the formaldehyde would no doubt be much more pronounced than the results suggest.

Summary and Conclusions.

Lipase can be conveniently estimated by titration of the acid formed from ethyl butyrate, when the reaction is carried out in a phosphate buffer medium.

The optimum p_H for the action of pancreatic lipase is determined by the substances present in the reacting mixture. With a purified pancreas powder containing lipase, the optimum p_H in phosphate and in phosphate-borax buffer solutions was at p_H 7·0, and in borax-boric acid buffer solutions there was an optimal zone about p_H 8·4. The lipase of a glycerol extract of pig's pancreas in phosphate buffer mixtures, acting on ethyl butyrate, showed maximum activity at p_H 7·8, but gave no definite p_H optimum.

The enzyme does not promote the hydrolysis of ethyl butyrate in the complete absence of activators, even if the medium has favourable hydrogen ion concentration.

Increasing concentrations of phosphates and bile salts have a similar effect on the activity of lipase. In both cases, when the values of the activities are plotted against increasing salt concentration, a curve is obtained showing

first, a rapid rise, then a plateau, followed by another rise. The form of the curve appears to be determined by both the buffer value of the solutions and a specific influence of a chemical nature on the enzyme.

Phosphates should be present in any system of activators intended for complete activation of pancreatic lipase.

Our results suggest that the protein structure which has been ascribed to lipase is characteristic of the substances associated with the enzyme rather than of the enzyme itself. The ester-hydrolysing properties of the enzyme have been definitely altered by changing the accompanying protein. The apparent destruction of lipase by trypsin is due, in part, to the removal by hydrolysis of proteins which activate the lipase.

Formaldehyde has no appreciable effect on pancreatic lipase action in concentrations less than 0·2 %.

We wish to acknowledge our indebtedness to Prof. Cohen, in whose laboratory and under whose guidance the investigation was carried out, to the Royal Society for a grant which covered part of the cost of the materials, and to the Department of Scientific and Industrial Research for grants which have enabled us to devote our whole time to the research.

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